Structural and Interaction Studies of Polysaccharides by NMR Spectroscopy

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Abstract

In this thesis, polysaccharides of different origins have been investigated by NMR spectroscopy with regard to structures and interactions.

In paper I, hydrogen bonding interactions in kappa- (κ) and kappa/mu- (κ/μ) hybrid carrageenan oligosaccharides were studied by NMR spectroscopy of hydroxy protons. Weak hydrogen bonding over the 1→4 glycosidic linkage of μ-carrabiose was observed in the κ/μ oligosaccharides. In the hexasaccharide κμκ and octasaccharide κμμκ, one and two hydrogen bonds respectively were found over the 1→4 glycosidic linkage. In the decasaccharide κμμμκ, a loss of a hydrogen bond in the inner μ-unit was observed, suggesting higher flexibility in a μ-unit having other μ-units as neighbors.

In papers II and III, the activities and substrate specificities of three new alginate lyases were characterized. The alginate lyase from the marine bacteria *Pseudomonas alginovora* showed an *endo*-cleaving activity with a strict specificity for M-block alginates. The AlyA1 lyase from the marine bacteria *Zobellia galactanivorans*, showed an *endo*-cleaving activity with a strict specificity for G-block alginates, whereas AlyA5 showed an *exo*-cleaving activity with a broad substrate tolerance, degrading oligosaccharides down to monosaccharides.

Paper IV describes the elucidation of the primary structure of the O-specific polysaccharide and core oligosaccharide from the lipopolysaccharide produced by the Gram-negative bacteria *Plesiomonas shigelloides* strain CNCTC 92/89 (O24:H8). The O-specific polysaccharide was composed of a tetrasaccharide repeating unit: →3)-α-Fuc*p*NAc-(1→3)-α-Gal*p*NAcA-(1→3)-α-Qui*p*NAc-(1→, with α-Rha*p*NAc linked (1→4) to α-Gal*p*NAcA. The core oligosaccharide was composed of a decasaccharide.

Keywords: NMR spectroscopy, carbohydrate, polysaccharide, alginate, alginate lyase, carrageenan, lipopolysaccharide, *Plesiomonas shigelloides*.

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Dedication

To the best family in the world – **My family!**

An expert is a person who has made all the mistakes that can be made in a very narrow field.

Niels Bohr

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Eric Morssing Vilén, Lena C. E. Lundqvist, Diane Jouanneau, William Helbert, and Corine Sandström (2010). NMR Study on Hydroxy Protons of κ- and κ-/μ-Hybrid Carrageenan Oligosaccharides: Experimental Evidence of Hydrogen Bonding and Chemical Exchange Interactions in κ/μ Oligosaccharides. *Biomacromolecules* 11, 3487–3494.

II Lena C. E. Lundqvist, Murielle Jam, Tristan Barbeyron, Mirjam Czjzek and Corine Sandström (2012). Substrate Specificity of the Recombinant Alginate Lyase from the Marine Bacteria *Pseudomonas alginovora*. *Carbohydrate Research* 352, 44-50.

III François Thomas, Lena C. E. Lundqvist, Murielle Jam, Alexandra Jeudy, Tristan Barbeyron, Corine Sandström, and Mirjam Czjzek (2013). Comparative Characterization of Two Marine Alginate Lyases from *Zobellia galactanivorans* Reveals Distinct Modes of Action and Exquisite Adaptation to Their Natural Substrate. *The Journal of Biological Chemistry* 288 (32), 23021-23037.

IV Lena C. E. Lundqvist, Marta Kaszowska, and Corine Sandström. Structural Study of the O-specific Polysaccharide and the Core Oligosaccharide from the Lipopolysaccharide Produced by *Plesiomonas shigelloides* O24:H8 (Strain CNCTC 92/89) (manuscript).

Papers I-III are reproduced with the permission of the publishers.

The contribution of Lena C. E. Lundqvist to the papers included in this thesis was as follows:

I Planning and discussion of the project together with the co-authors. Part of the experimental work, data analysis and writing.

II Discussion of the project together with the co-authors. Planning and execution of all experimental work (NMR, SEC and MS), analysis of the data and the majority of the writing.

III Discussion of the project with the co-authors. Planning and execution of all NMR and MS related experimental work. Analysis and writing of the NMR and MS related results.

IV Initiation and planning of the project. All experimental work, analysis of the data and majority of the writing.

Abbreviations

BLAST	Basic Local Alignment Search Tool
CHCA	α-cyano-4-hydroxycinnamic acid
CNCTC	Czechoslovak National Collection of Type Cultures
COSY	COrrelation SpectroscopY
CPMAS	Cross Polarisation Magic Angle Spinning
D	α-D-galactopyranose when referring to carrageenans
DA	3,6-anhydro-α-D-galactopyranose when referring to carrageenans
DEH	4-deoxy-L-*erythro*-5-hexoseulose uronic acid
DHB	2,5-dihydroxybenzoic acid
DP	Degree of Polymerization
dδ/dT	Temperature coefficient
EI	Electron Ionization
ESI	Electro-Spray Ionization
FID	Flame Ionization Detector
Fuc	Fucose
FucNAc	*N*-acetylfucosamine
G or Gal	Galactopyranose when referring to carrageenans
G or GulA	Guluronic acid when referring to alginates
GalA	Galacturonic acid
GalN	Galactosamine
GalNAc	*N*-acetylgalactosamine
GalNAcA	*N*-acetylgalactosamine acid
GC	Gas Chromatography
Glc	Glucose
GlcN	Glucosamine
HILIC	Hydrophilic Interaction Liquid Chromatography
HMBC	Heteronuclear Multiple Bond Correlation spectroscopy
HPLC	High-performance liquid chromatography

HRMAS	High Resolution Magic Angle Spinning
HSQC	Heteronuclear Single-Quantum Coherence spectroscopy
Kdo	3-deoxy-D-*manno*-oct-2-ulosonic acid
L,D-Hep	L-*glycero*-D-*manno*-heptose
LPS	LipoPolySaccharide
M	Mannuronic acid
M/G-ratio	Mannuronic acid/Guluronic acid - ratio
m/z	mass to charge ratio
MALDI	Matrix-Assisted Laser Desorption Ionization
MD	Molecular Dynamics
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser Effect
NOESY	Nuclear Overhauser Effect SpectroscopY
OS	OligoSaccharide
PGC	Porous Graphite Chromatography
ppm	parts per million
PS	PolySaccharide
Q	Quadrupole
QuiNAc	*N*-acetylquinovosamine
RhaNAc	*N*-acetylrhamnosamine
ROE	Rotating-frame nuclear Overhauser Effect
ROESY	Rotating-frame nuclear Overhauser Effect SpectroscopY
SEC	Size-Exclusion Chromatography
THAP	Trihydroxyacetophenone
TOCSY	TOtal Correlation SpectroscopY
TOF	Time-Of-Flight
Tris	2-amino-2-hydroxymethyl-propane-1,3-diol
UV	UltraViolet
WATERGATE	Water suppression by gradient tailored excitation
δ	Chemical shift
$\Delta\delta$	Chemical shift difference
Δ	4-deoxy-L-*erythro*-hex-4-ene pyranosyluronate

1 Introduction

Polysaccharides are carbohydrate polymers and are among the most ubiquitous and versatile biomaterials available. They have a high structural diversity which is a result of the high variability in monosaccharide composition, sequence and linkage position, anomeric configuration, molecular weight and charge density. Further variability arises with their susceptibility to ionic strength and degree of hydration, which can alter the higher-order structure. The structural diversity of polysaccharides is a reason for the immense variance of chemical properties they possess. In nature polysaccharides are found, for example, as plant building material (cellulose), as energy storage (starch) or as protective coating for bacteria. In industry carbohydrate polymers are used in various areas such as food, textile, paper, pharmaceutical, adhesives, and biofuel.

Alginates and carrageenans are two marine polysaccharides studied in this thesis. They are used predominantly in the food industry as viscosity enhancers. Depending on their structure, these polysaccharides possess different degrees of gel-forming ability. The fine structure of the polysaccharides and how alteration of the structure affects the chemical properties are important for the optimization of the gel-forming ability. The carrageenan polysaccharides may be altered through alkali treatment to increase the gel strength, but the extent to which this should be done to obtain the best gelation properties is in many cases not yet optimized. In the case of alginate polysaccharides no chemical conversion of mannuronic acid into guluronic acid can be done, but there is ongoing research in finding enzymatically modification techniques to alter the structure to achieve the optimal gelation ability.

Lipopolysaccharides (LPS) are the main component of the outer membrane of Gram-negative bacteria. The development of vaccines against Gram-negative bacteria is based on the production of antibodies that can bind to the outer part of the lipopolysaccharide. The elucidation of the polysaccharide structure is essential for the construction of antibodies.

1.1 Specific Aims

In this thesis, polysaccharides of different origins have been studied with respect to structures and interactions.

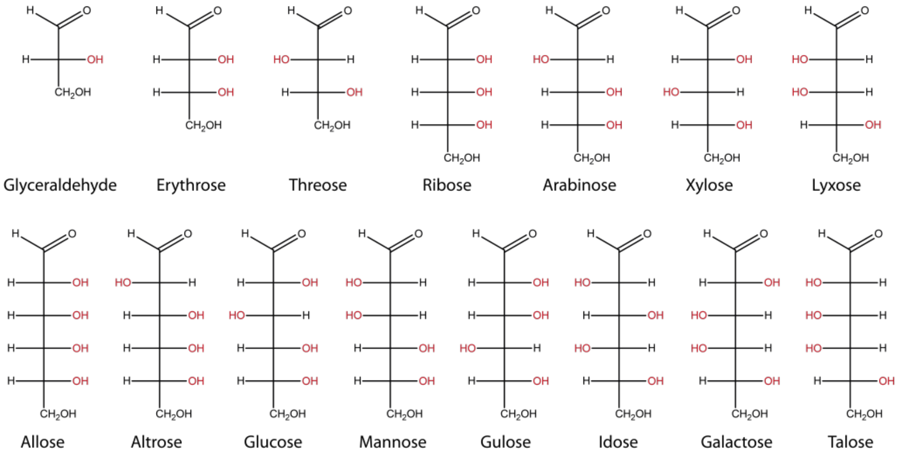
In paper **I**, the hydration, hydrogen bonding, and structural flexibility of kappa, and kappa/mu hybrid carrageenan oligosaccharides were studied using NMR of hydroxy protons.

In papers **II** and **III**, three new alginate lyases from the two marine bacteria *Pseudomonas alginovora* and *Zobellia galactanivorans* were characterized in terms of substrate specificity, end products and minimal recognition pattern.

In paper **IV**, the primary structure of the O-specific polysaccharide and of the core oligosaccharide from the lipopolysaccharide produced by *Plesiomonas shigelloides* O24:H8 (Strain CNCTC 92/89) were elucidated.

2 Carbohydrates

Carbohydrates were first described as hydrates of carbon because of their general empirical formula $C_n(H_2O)_n$ but they are more properly defined as polyhydroxy aldehydes, polyhydroxy ketones, or derivatives of these (Kamerling *et al.*, 2007; McNaught, 1997). Carbohydrates are divided into monosaccharides, oligosaccharides and polysaccharides depending on the number of residues. Monosaccharides are the single carbohydrate building blocks, whereas oligosaccharides are composed of ~2-10 monosaccharides and polysaccharides of more than ten monosaccharides. The monosaccharides are described as aldoses or ketoses, based on whether they contain an aldehyde or a ketone functional group. They are further categorized according to the number of carbon atoms they contain in the backbone; trioses (3 carbons), tetroses (4), pentoses (5), hexoses (6), and heptoses (7). The acyclic forms of the D-aldoses containing three to six carbons are illustrated in Figure 1.

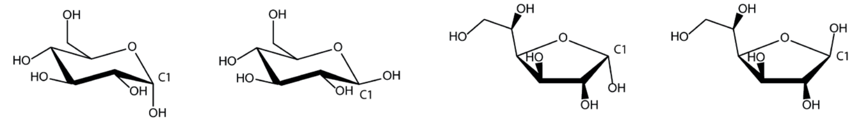


Glyceraldehyde Erythrose Threose Ribose Arabinose Xylose Lyxose

Allose Altrose Glucose Mannose Gulose Idose Galactose Talose

Figure 1. Fischer projections of the D-enantiomers of the common aldotriose, aldotetroses, aldopentoses and aldohexoses.

The overall configuration of the monosaccharide is defined as D or L, and is determined by the orientation of the OH-group at the highest numbered stereogenic carbon. The monosaccharides exist preferably as cyclic hemiacetals and hemiketals, and the cyclic forms arise from the intramolecular nucleophilic attack of a hydroxyl oxygen atom at the carbonyl carbon of the acyclic species. This cyclization can result in a five-membered furanose ring (*f*) or a six-membered pyranose ring (*p*) (Figure 2). This ring formation creates a new stereogenic center at the carbonyl carbon, called anomeric center. There are two possible stereoisomers referred to as anomers, and they are designated as α or β depending on the spatial orientation of the anomeric hydroxyl group (Figure 2). A mixture of the chiral forms of α and β is represented by a "wavy" line. The interconversion between the α and β-forms of pyranose and furanose, through ring opening and closure until the equilibrium is reached is referred to as mutarotation.



α-D-glucopyranose (α-D-Glc*p*) β-D-glucopyranose (β-D-Glc*p*) α-D-glucofuranose (α-D-Glc*f*) β-D-glucofuranose (β-D-Glc*f*)

Figure 2. The four different ring structures of D-glucose. Anomeric carbon at C1.

Furanose and pyranose ring forms can exist as different conformers that can interconvert if the energy barrier is small. A furanose can exist in two different families of conformers; twist (T) and envelope (E). A pyranose can exist in five different families of conformers: chair (C), boat (B), skew (S), half-chair (H) and envelope (E) (Figure 3). The most stable conformation of a six-membered pyranose ring is the chair; there are two isomeric chair conformations, the $^4C_1$ (the superscript corresponds to the number of the C-atom in the upper position of the chair and the subscript to that in the lower position) and $^1C_4$ (Figure 3). The conformation that is preferred is the one that allow most of the bulky groups (hydroxyl and hydroxymethyl groups) to be in the equatorial positions.

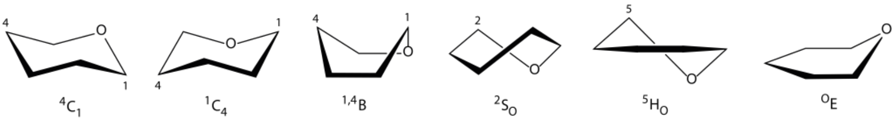


Figure 3. The two chair (C) conformers $^4C_1$ and $^1C_4$, one boat (B), one skew (S), one half-chair (H), and one envelope (E) conformers.

Monosaccharides such as the ones presented in Figure 1, can be modified into sugar derivatives that cannot be represented by the general formula $C_n(H_2O)_n$. The most common derivatives are the sugar acids, deoxy sugars, amino sugars and amino sugars with *N*-acetylation (Figure 4).

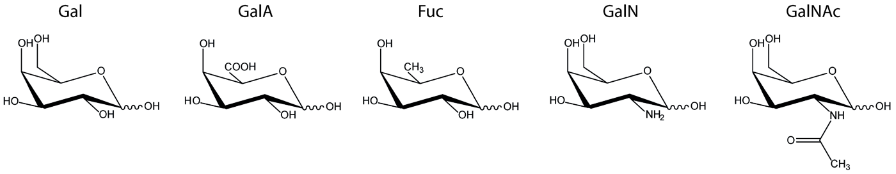


Figure 4. Examples of different monosaccharide derivatives of galactose. From the left to the right: Gal (galactose), GalA (the uronic acid of galactose), Fuc (the C6 deoxy sugar of galactose), GalN (the C2 amino sugar of galactose), and GalNAc (the acetylated amino sugar of galactose).

Monosaccharides can be covalently joined together by a glycosidic linkage to form oligo- or polysaccharides. The glycosidic linkage is formed when a hemiacetal group of one monosaccharide reacts with any of the free hydroxyl groups of another monosaccharide through a condensation reaction, resulting in an acetal. To describe the location of the glycosidic linkage, position numbers are used, for example (1→3) linked if the anomeric carbon in an aldose reacts with the free hydroxyl group at carbon 3 of another sugar residue. In oligo- and polysaccharides, the end that contains the free anomeric carbon is called reducing end while the other end is called the non-reducing end. The size of the oligo- and polysaccharide can be described as the degree of polymerization (DP) that is defined as the number of monomeric units or the number of repeating units. Polysaccharides can be further classified as homopolysaccharides that are composed of identical monosaccharides or heteropolysaccharides that are composed of two or more different types of monosaccharides. The heteropolysaccharides can also be described as copolymers, with an alternating or block structure of the different monosaccharides. The oligo- and polysaccharides can furthermore have a linear or a branched structure. The high diversity within carbohydrates can be understood when looking at the variety of monosaccharide structures, the sequence of glycosidic linkages between the monosaccharides, the variability of linkages and the different kinds of branching. The function and properties of oligo- and polysaccharides are dependent on the structure, and with the high diversity the functions of polysaccharides are extensive.

In the following sections, the three different polysaccharides studied in this thesis are described in more detail.

2.1 Carrageenans

Carrageenan is the generic name for a family of polysaccharides found in several species of marine red algae (*Rhodophyceae*), where it is the main component of the cell wall and has both structural and signaling roles (Potin *et al.*, 1999; Kloareg & Quatrano, 1988). Carrageenans are composed of linear, sulfated chains of alternating 1→4 linked β-D-galactopyranose and 1→3 linked α-D-galactopyranose or 1→3 linked 3,6-anhydro-α-D-galactopyranose. The most common forms of carrageenans are traditionally classified by a Greek prefix; Iota (ι), Kappa (κ), Lambda (λ), Mu (μ), Nu (ν) and Theta (θ), where the three most commercially relevant carrageenans are ι-, κ-, and λ-carrageenan (Figure 5). The classification is based on the solubility in potassium chloride solution of the carrageenan polysaccharide (Smith & Cook, 1953), which is dependent on the position and number of sulfate groups and the presence of 3,6-anhydro bridges. The Greek prefix only describe the "ideal" dimeric structure, however natural carrageenans are nonhomogeneous macromolecules, and to describe the more complex structure a letter code based nomenclature is used (Knutsen *et al.*, 1994) (Figure 5). The 1→4 linked β-D-galactopyranose is denoted G, the 1→3 linked α-D-galactopyranose is denoted D, and the 1→3 linked 3,6-anhydro-α-D-galactopyranose is denoted DA. The number in front of the letter S defines the position of sulfation (Figure 5).

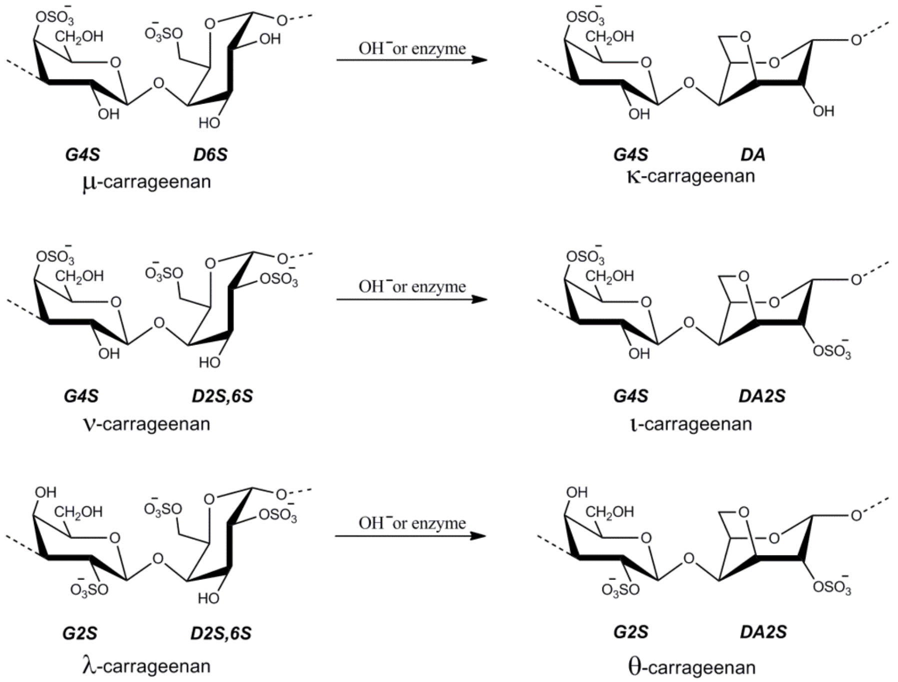


Figure 5. Schematic representation of the ideal dimeric units of the most commercially relevant carrageenans. The letter codes refer to the nomenclature developed by Knutsen *et al.* (1994).

The chemical structure depends on from which species of *Rhodophyceae* the carrageenan is extracted, on harvest season, on growing conditions and on the life stage of the algae (Hilliou *et al.*, 2012; Rinaudo, 2008). κ-Carrageenan is mainly obtained from *Eucheuma cottonii*, whereas ι-carrageenan is obtained from *Eucheuma spionosum* and λ-carrageenan from different species of *Gigartina* and *Chondrus*. In general terms κ-carrageenans are forming brittle and hard gels whereas ι-carrageenans are forming flexible and soft gels and λ-carrageenans will not form gels (van de Velde & De Ruiter, 2002). The difference in gel forming ability is related to the presence of anhydro bridge in the DA galactopyranosyl unit. This unit has a $^1C_4$ conformation that allows formation of a helical secondary structure, which is necessary for the gel formation. Carrageenans give gels through the formation of multiple helices and through the cation dependent aggregations between helical strands (van de Velde *et al.*, 2002b; Viebke *et al.*, 1995) (Figure 6).

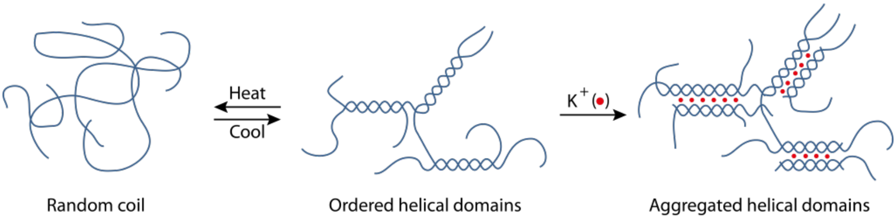


Figure 6. Model of carrageenan gelation. Random coil transformation upon cooling to ordered double helical domains. Further cooling or introduction of metal ions promote aggregation of the helical domains to form cross linked junction domains (Viebke *et al.*, 1995).

The biological precursors of κ- and ι-carrageenans, μ and ν respectively (Figure 5) are, as the λ-carrageenan, lacking the anhydro bridge, which results in that the D-unit has a $^4C_1$ conformation and causes 'kinks' in the regular chain. This prevents the formation of the helical strands and therefore prevents gelation (van de Velde *et al.*, 2002a). Native carrageenan always contains a certain amount of the biological precursor, which has a negative effect on the gelling ability. In the industrial processing of carrageenans, the precursor is transformed into κ- and ι-carrageenans by alkali treatment to obtain better gel forming properties (Campo *et al.*, 2009; Falshaw *et al.*, 2001). In order to study the alkali conversion of the precursor, and the effect that this conversion has on the physical properties, the fine structure of the native carrageenan has to be determined. This can be done by enzymatic degradation with carrageenases followed by studies of the end products (Jouanneau *et al.*, 2010). To describe dimeric unit with G- or D/DA-reducing end, they are designated as carrabiose or neocarrabiose, respectively.

The viscosity of a carrageenan is not only dependent on the type of carrageenan, but also on the molecular weight, temperature, concentration and ion content (Rinaudo, 2008). The industrial use of carrageenans is mainly as stabilizers, thickeners or gelling agents in food applications where it is known as E407 (carrageenan) and E407a (processed eucheuma seaweed). The average molecular weight of the commercial carrageenans is between 400-600 kDa.

2.2 Alginates

Alginate is a collective name for a family of polysaccharides mainly found in marine brown algae (*Phaeophyceae*) where it is a structural component. Alginates are linear unbranched copolymers composed of 1→4 linked β-D-mannuronic acid and its C5 epimer, α-L-guluronic acid (Figure 7A). These copolymers are composed of block structures that are varying in both composition and sequence. The different blocks are composed of homopolymeric regions of mannuronic acid (M) and guluronic acid (G) and of alternating heteropolymeric regions (MG-blocks) (Haug *et al.*, 1967). The two monomers differ in the configuration at C5, and adopt different chair conformations. Guluronic acid favors the $^1C_4$ conformation and mannuronic acid the $^4C_1$ conformation, allowing the carboxyl group to be in an equatorial position. The differences in conformation of the two sugars result in that alginates can contain four different glycosidic linkages: diequatorial (MM), diaxial (GG), equatorial-axial (MG), and axial-equatorial (GM). The diaxial linkage in guluronic acid will lead to the formation of a buckled chain (Figure 7C) and the diequatorial linkage in mannuronic acid will form a flat ribbon structure (Figure 7D) (Rees, 1972).

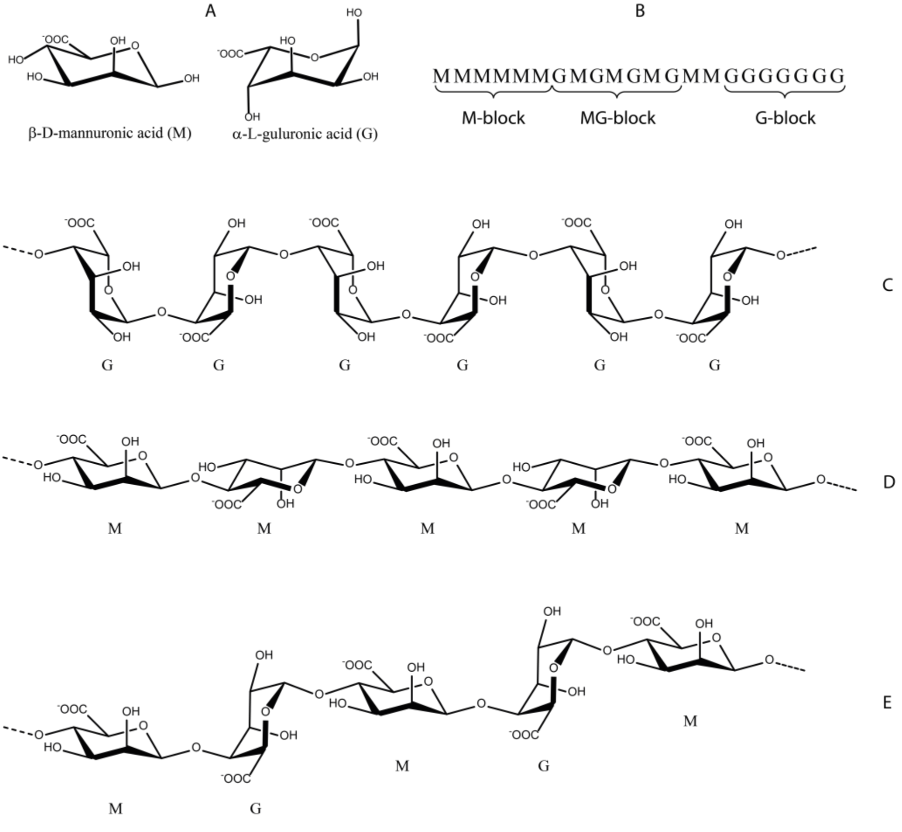


Figure 7. Structural characteristics of alginate. (A) alginate monomers, (B) block distribution, (C) chain conformation in G-blocks, (D) chain conformation in M-blocks, (E) chain conformation in alternating MG-blocks.

Alginates are extracted from different *Laminaria*, *Macrocystis* and *Ascophyllum* species (Gacesa, 1988). The chemical composition and sequence of monosaccharides depend on the species from which the alginate is extracted, the season for the harvest, the age and the type of tissue it is extracted from. (Lee & Mooney, 2012; Indergaard & Skjåk-Braek, 1987). Alginates can form gels in two different ways, either through so called acid gels or through ionic cross-linking (Draget *et al.*, 1996; Rees, 1972). The acid gels are not so extensively studied, but alginates form gel at pH values below the pK_a value of mannuronic acid (pKa 3.38) and guluronic acid (pKa 3.65) (Smidsrød *et al.*, 1969). The gels with industrial use are the ionic cross-linked alginates. It is the ion binding properties of the alginates that are central for the gelling properties. Alginates with a high content of G-blocks are known to form strong and brittle gels whereas alginates with a higher content of M-blocks form weaker and more flexible gels. All types of alginate block structures bind divalent cations through their polyanionic nature. However the G-block stretches show a higher affinity to ions in increasing order of $Mg^{2+} < Ca^{2+} < Sr^{2+} < Ba^{2+}$ (Haug & Smidsrød, 1965). This is because G-block stretches are able to chelate these ions and form gels through a ribbon ordered conformation known as the "egg-box" model (Figure 8) (Grant *et al.*, 1973; Rees, 1972). The ratio between the content of mannuronic acid and guluronic acid (M/G-ratio) can be used to predict the nature of the gel that will be formed in the presence of divalent cations.

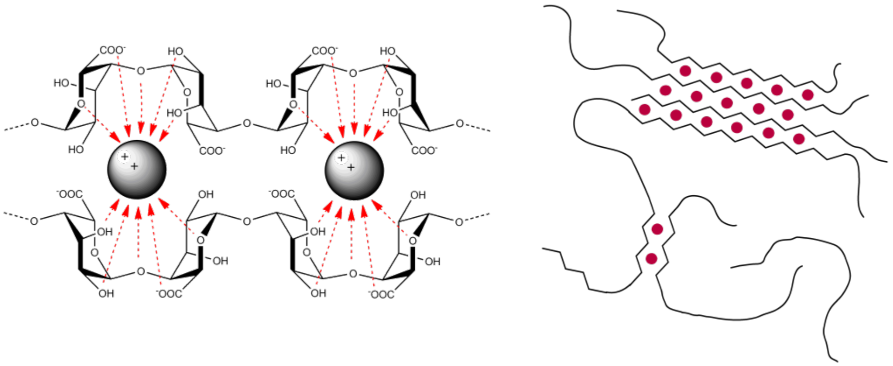


Figure 8. The egg-box model shows how the divalent cations fit into the G-block structure like eggs in a box. Alginate hydrogels are formed through ionic cross-linking of these G-block regions.

This variance in gelling makes it interesting to be able to produce alginate with a higher content of G-blocks, and thereby improving the gelling capacity. The conversion of M into G can be made enzymatically by a C5 epimerase (Gacesa, 1987) (Figure 9). One commonly used method to determinate the M/G-ratio and the diad (disaccharide unit) and triad (trisaccharide unit) composition is NMR analysis (Grasdalen, 1983). One problem with this analysis is that the samples have to be partially hydrolysed to reduce the viscosity which can result in alteration of the sample. A nondestructive way of analyzing the alginate polymer is to use either diffusion (Morssing-Vilén *et al.*, 2011), HRMAS or CPMAS (Salomonsen *et al.*, 2009) NMR. Alginates are also found in soil bacteria as capsular polysaccharides. The structural difference of alginates found in soil bacteria compared to the alginates in algae is that the mannuronic acid can be acetylated at O2, and O3 at varying degree. The industrial use of alginates is mainly as stabilizers, thickeners or gelling agents in food applications where they are known under the EU additive E-numbers E400-E405 (alginic acid, sodium alginate, potassium alginate, ammonium alginate, calcium alginate and propane-1,2-diol alginate).

2.2.1 Alginate lyases

Alginate lyases catalyze the degradation of alginates by cleaving the glycosidic bond through a β-elimination reaction generating an unsaturated hexenuronic acid residue (Δ) at the non-reducing end and a new reducing end at the point of cleavage (Gacesa, 1987) (Figure 9). Alginate lyases can be found in marine algae, marine mollusks, and in microorganisms (Wong *et al.*, 2000). They are classified according to their substrate specificities where M-lyases (EC 4.2.2.3) have a preference for M-blocks and G-lyases (EC 4.2.2.11) have a preference for G-blocks. The classification of lyases is confusing because almost all lyases cleave more than one of the four possible glycosidic linkages M-M, M-G, G-M or G-G but at different rates (Wong *et al.*, 2000).

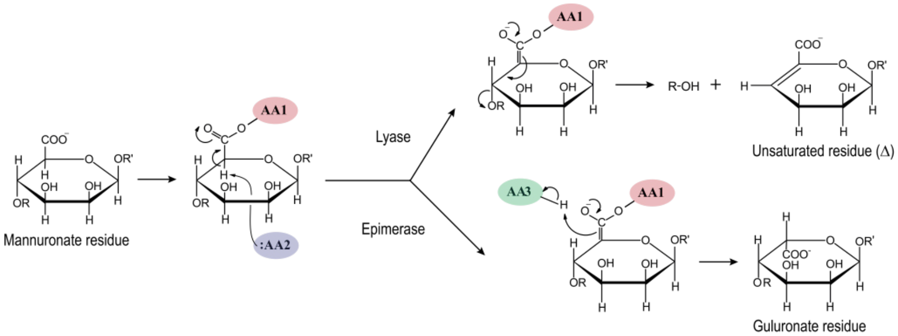


Figure 9. A unified mechanism for alginate lyase and C5 epimerases. AA1-AA3 refers to the amino acid residues on the enzyme (Gacesa, 1987).

Alginate lyases are also classified as *endo*- or *exo*- enzymes based on their mode of action. An *endo*-acting enzyme cleaves the polysaccharide in the middle of the chain whereas an *exo*-acting enzyme acts on unsaturated and saturated polymers from the reducing or non-reducing end to give mono-, di- or longer oligosaccharides. A cleavage is resulting in the new unsaturated non-reducing end, 4-deoxy-L-*erythro*-hex-4-ene pyranosyluronate (denoted as Δ). The Δ monosaccharide can be spontaneously converted to 4-deoxy-L-*erythro*-5-hexoseulose uronic acid (DEH) (Figure 10).

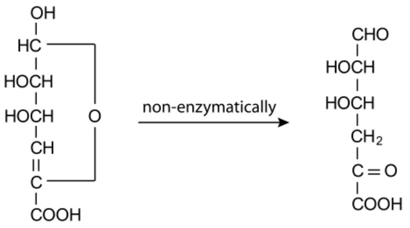


Figure 10. Non-enzymatic conversion of the monosaccharide 4-deoxy-L-*erythro*-hex-4-ene pyranosyluronate (Δ) to 4-deoxy-L-*erythro*-5-hexoseulose uronic acid (DEH).

The majority of the alginate lyases studied are M-lyases with an endolytic activity, and most of the products obtained range from dimers to pentamers (Sutherland, 1995). Alginate lyases are not yet produced for commercial applications, but are used in the research area.

They can be used to determine the fine structure of alginates as well as the mode of action of the C5 epimerase. The information about the terminal non-reducing end of the end product is lost because the same residue (Δ) is formed whichever M or G residue is cleaved by the lyase, which leads to difficulties in determining the exact specificity of the enzymes (Sutherland, 1995).

Through the use of alginate lyases, there is a hope to be able to develop a third-generation biofuel by the use of marine biomass (Wargacki *et al.*, 2012; Takeda *et al.*, 2011).

2.3 Lipopolysaccharides

Bacterial lipopolysaccharides (LPS) are the main component of the outer membrane of Gram-negative bacteria. Lipopolysaccharides normally consists of a hydrophobic domain known as lipid A, a non-repeating oligosaccharide (core), and a distal polysaccharide (O-specific) (Figure 11) (Caroff & Karibian, 2003; Erridge *et al.*, 2002; Raetz & Whitfield, 2002).

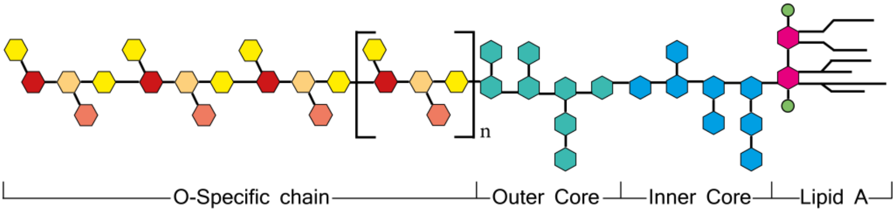


Figure 11. Schematic illustration of a lipopolysaccharide structure.

The lipid A has a rather conserved architecture, and is generally composed of phosphorylated glucosamine and fatty acids. The core is linked to the lipid A through a linkage between Kdo (α-3-deoxy-D-*manno*-oct-2-ulosonic acid) and GlcN. This bond is sensitive to mild acidic hydrolysis. The core is divided into two different regions, the inner and the outer core. The inner core, closest to the lipid A, is more conserved and consists of unusual sugars such as Kdo and L-*glycero*-D-*manno*-heptose (L,D-Hep) (Figure 12), whereas the outer core contains more common sugars and shows a higher diversity.

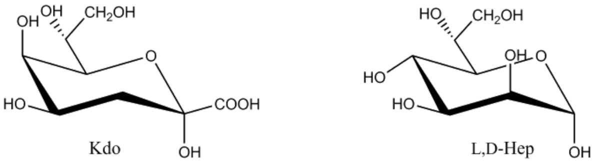


Figure 12. Structure of 3-deoxy-α-D-*manno*-oct-2-ulosonic acid (Kdo) and L-*glycero*-α-D-*manno*-heptopyranose (L,D-Hep).

The O-specific polysaccharide, consisting of repeating oligosaccharide units is attached to the outer core and shows the highest structural variety. This variety comes from that the repeating unit often contains between three and eight monosaccharides which not only differ in the type of sugar residue but also in sequence, linkage and substitution. The O-specific polysaccharide is usually considerably longer than the core polysaccharide.

The lipid A, core and O-specific regions are all significant for the biological activity and involvement in host-bacterium interactions. The toxicity of the LPS depends mainly on the chemical structure of lipid A and is only modulated by the core and O-specific polysaccharide. The core and O-specific oligosaccharide are essential for the biological and physical properties of the lipopolysaccharide and play a significant role in interactions with the host. LPS are known to be either of smooth type (S) or rough type (R). The smooth type LPS contains an O-specific chain, whereas the rough form lacks the O-specific chain. This O-specific chain is protecting the bacteria from the effect of antibiotics. There is a hope to be able to develop vaccines against Gram-negative bacteria. This could be achieved by production of antibodies that can bind to the lipopolysaccharide. To be able to create such an antibody the lipopolysaccharide structure has to be known.

2.3.1 *Plesiomonas shigelloides*

Plesiomonas shigelloides is a Gram-negative, rod-shaped bacterium, which has recently been relocated from the *Vibrionaceae* family to the *Enterobacteriaceae* family (Farmer *et al.*, 2006; Stock, 2004). *P. shigelloides* shows only 8 % and 7 % genetical similarities to the *Enterobacteriaceae* and *Vibrionaceae* family respectively, even though they share biochemical and antigenic properties (Ruimy *et al.*, 1994).

P. shigelloides is an opportunistic pathogen that causes gastrointestinal illness, with diarrhea as the main symptom. The primary habitat of *P. shigelloides* is fresh water ecosystems. Infections with *P. shigelloides* are related to drinking untreated water or eating uncooked shellfish. The pathogenicity of *P. shigelloides* is not entirely understood, though they are producing a number of potential virulence factors including cholera like toxins (Gardner *et al.*, 1987), thermostable and thermolabile toxins (Sears & Kaper, 1996), β-hemolysin (Janda & Abbott, 1993), and cytotoxin complexes (Okawa *et al.*, 2004), where the lipopolysaccharide is one of the potential virulent factors.

The strains found so far from *P. shigelloides* have been classified into 102 O serotypes and 50 H serotypes, based on their O-antigen and H-antigen in the form of flagellar proteins, respectively (Aldová & Shimada, 2000). The complete structure of the LPS from *P. shigelloides* is only known for three strains, serotype O54:H2 strain CNCTC 113/92 (Lukasiewicz *et al.*, 2006b; Niedziela *et al.*, 2002; Czaja *et al.*, 2000), serotype O74:H5 strain CNCTC 144/92 (Lukasiewicz *et al.*, 2006a; Niedziela *et al.*, 2006) and serotype O37 strain CNCTC 39/89 (Kaszowska *et al.*, 2013a). The core structure substituted with an O-specific polysaccharide is determined for the serotype O1 strain 302-73 (Pieretti *et al.*, 2010; Pieretti *et al.*, 2009; Pieretti *et al.*, 2008). The core structure has been determined for the three strains, serotype O33 strain CNCTC 34/89 (Nestor *et al.*, 2014), serotype O17 strain 7-63 (Kubler-Kielb *et al.*, 2008), strain PCM 2231 (Maciejewska *et al.*, 2013) and serotype O13 strain CNCTC 80/89 (Kaszowska *et al.*, 2013b). The O-specific polysaccharide structure has been resolved for four strains, serotype O51 strain CNCTC 110/92 (Maciejewska *et al.*, 2009), strain AM36565 (Säwén *et al.*, 2012), strain 22074 and 12254 (Linnerborg *et al.*, 1995). Many of the structures are reviewed by Nazarenko et al. (Nazarenko *et al.*, 2011).

It has been found that the *Plesiomonas* genus does not have a uniform core structure, as many other genera have. There are however some similarities in the structures, such as *P. shigelloides* lack the charged phosphorous containing groups in the inner core structure. Some of the LPS from *P. shigelloides* have been obtained in a higher degree in the phenol phase rather than the usual water phase, from hot phenol/water extraction, which can be explained by the presence of deoxy sugar, deoxyamino sugar and the presence of *O*-acetyl groups in the O-antigen polysaccharide (Aquilini *et al.*, 2013; Pieretti *et al.*, 2008).

3 Experimental

3.1 Nuclear magnetic resonance (NMR) spectroscopy

Nuclear magnetic resonance spectroscopy is one of the most powerful analytical tools for structure elucidation of carbohydrates. Both structure and conformation of the carbohydrate can be determined from ^{1}H and ^{13}C one-dimensional (1D) NMR experiments together with different two-dimensional (2D) NMR experiments. It is however still problematic to resolve all structures due to the structural diversity of carbohydrates and the limited chemical shift dispersion.

3.1.1 Structural analysis by NMR

Carbohydrates have some characteristic parameters that are advantageous for structural elucidation and conformational analysis. The anomeric resonances for the protons are generally found in the chemical shift range of 4.2-5.8 ppm, with the α-anomeric protons between 4.8-5.8 ppm and the β-anomeric protons between 4.2-4.8 ppm. The anomeric resonances for the carbons are found in the chemical shift range of 95-110 ppm, with the α-anomeric carbons normally around 98-103 ppm, and the β-anomeric carbons around 103-106 ppm. This, together with the scalar coupling constants $^{3}J_{\mathrm{H1,H2}}$ can give information about the anomeric configuration. The $^{3}J_{\mathrm{H1,H2}}$ for sugar residues in the *gluco*- or *galacto*-configuration is around 3-4 Hz for the α-form and 7-8 Hz for the β-form. For sugars in the *manno*-configuration, the $^{3}J_{\mathrm{H1,H2}}$ is small; around 2 Hz for the α-form and even smaller for the β-form with the result that they are not always resolved (Bubb, 2003). When the $^{3}J_{\mathrm{H1,H2}}$ coupling constants are too small and/or not resolved, the $^{1}J_{\mathrm{C1,H1}}$ coupling constant can be used, a value of 170 Hz indicates the α-form, while 160 Hz is an indication for the β-form.

The ring protons are generally found in the 3.2-4.5 ppm region. The methyl groups from 6-deoxy sugars and from *O*- and *N*-acetyl groups are found in the range of 1.2-2.3 ppm. The ring carbons will be found in the range of 50-85 ppm, where carbons with nitrogen attached will appear at around 50-60 ppm, unsubstituted carbons in the range of 65-75 ppm. Carbons involved in glycosylation will exhibit a large downfield shift of around 5-10 ppm. Unsubstituted C6 carbons are found around 60-63 ppm and C6 carbons linked to another sugar residue will appear around 65-70 ppm. The carbon signals from methyl groups are found in the range of 15-30 ppm, and carbonyl carbons are found at 165-185 ppm. These characteristic ^{1}H and ^{13}C chemical shifts are shown in Figure 13. How the different NMR experiments are used and the kind of information that can be obtained from them are described in the following section.

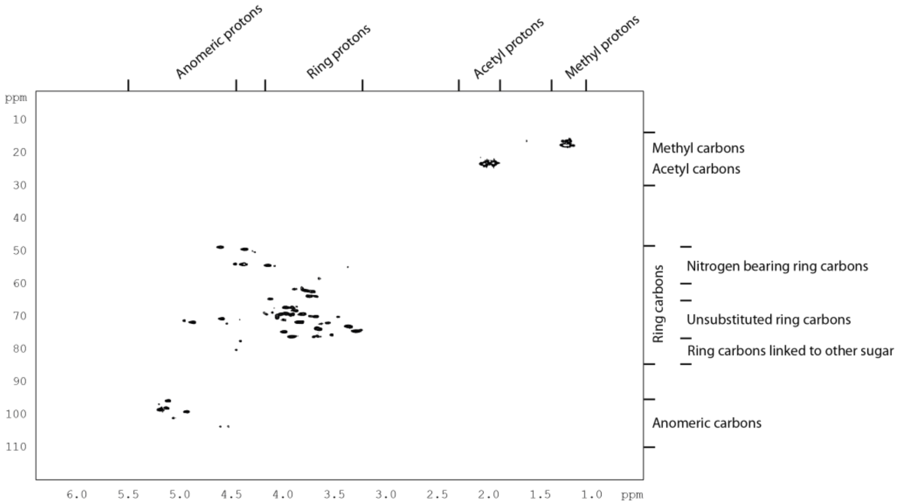


Figure 13. Schematic HSQC spectrum showing the typical chemical shift ranges of NMR signals from protons and carbons in carbohydrates.

One-dimensional ^{1}H and ^{13}C NMR experiments.

1D ^{1}H NMR spectroscopy is often the first step in structural studies by NMR. The number of sugar residues can be estimated by integration of the signals in the region of the anomeric protons. The number of anomeric resonances in the 1D ^{13}C NMR spectrum will further confirm these results.

^{1}H and ^{13}C 1D experiments can also give some indication about the linkage and sequence of the sugar residues through changes in chemical shifts, but in general both homo- and heteronuclear 2D NMR experiments are required to fully assign the structure of oligo- and polysaccharides.

If the anomeric signals are well resolved they appear as doublets from which the $^{3}J_{H1,H2}$ coupling constants can be obtained, and give information about the anomeric configuration. ^{13}C NMR is much less sensitive than ^{1}H NMR due to the low natural abundance of the ^{13}C nucleus and the fact that the gyromagnetic ratio is only ¼ of that of ^{1}H, but the ^{13}C spectra show a greater dispersion of chemical shifts.

Homonuclear through-bond correlations

^{1}H-^{1}H COSY (correlation spectroscopy) is used to establish the direct neighboring connectivity of protons. The anomeric protons are often used as starting point in the assignment of the ring protons due to the fact that they have well separated chemical shifts and are generally only coupled to one proton. The connectivity between protons within a sugar residue can from this be mapped out via a series of cross-peaks (Figure 14). There can be difficulties in assigning all protons, due to overlapping signals or lack of cross-peaks due to small ^{3}J coupling constants.

^{1}H-^{1}H TOCSY (total correlation spectroscopy) is related to COSY in the way that cross-peaks of coupled protons are detected. The additional information from the TOCSY spectrum is that there are cross-peaks between (almost) all spins in the spin system (Figure 14). The magnetization is transferred, during the mixing time, to the vicinal coupling partner, and can be further transferred throughout the entire spin system. The magnetization transfer can be interrupted by small ^{3}J coupling constants. This property is advantageous when deducing the configuration to be *manno*, *galacto* or *gluco* (Gheysen *et al.*, 2008). The number of transfer steps can be adjusted by changing the mixing time, a mixing time of 20 ms (one step transfer) will give essentially the same information as the COSY experiment, whereas a mixing time of 80-120 ms will give a five to six steps transfer.

Selective 1D TOCSY is a variant of the 2D experiment. It is performed by selective excitation of just one or a few resonances in the spectrum, followed by a TOCSY transfer. This results in a 1D spectrum that is a sub-spectrum of the full 2D TOCSY spectrum, showing just those resonances correlated with the selectively excited peak(s).

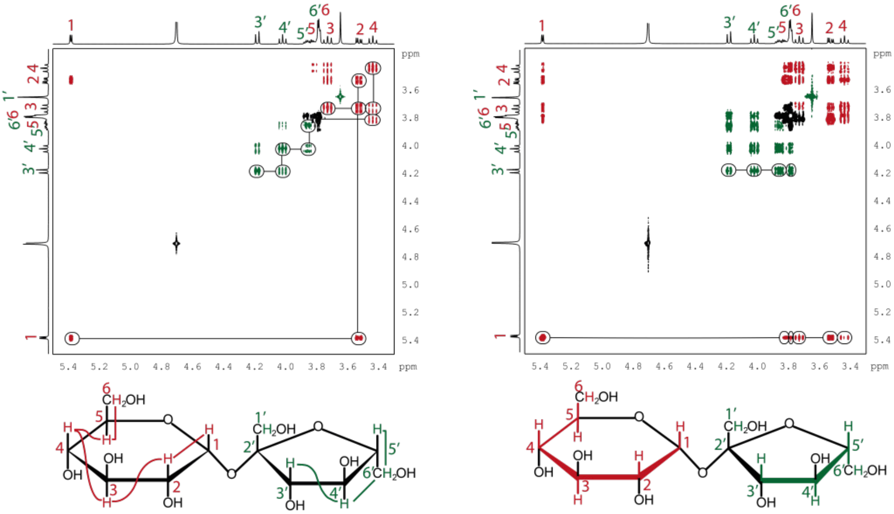


Figure 14. COSY (left) and TOCSY (right) spectra of sucrose, showing the two separated spin systems of glucose (red) and fructose (green). In the COSY spectrum the spin system is revealed stepwise, whereas the complete spin system is revealed in the TOCSY spectrum.

Heteronuclear through-bond correlations

^{1}H-^{13}C HSQC (heteronuclear single quantum correlation) provides proton carbon coupling across a single bond and correlates the protons with the directly bonded carbons, through one bond couplings. The cross-peaks contain information about the chemical shifts of the corresponding protons and carbons. The number of monosaccharides can more easily be determined in an HSQC spectrum than in a ^{1}H spectrum due to the added dispersion of chemical shifts also in the carbon dimension. Using multiplicity edited HSQC experiment, it is possible to discriminate between CH/CH$_3$ groups that give positive signals and CH$_2$ groups that give negative signals (Figure 15). Quaternary carbons, lacking directly attached protons will not be visible in this experiment (Figure 15). The $^1J_{CH}$ coupling constants can be obtained from a non-decoupled HSQC NMR experiment and give information about the anomeric configuration.

^{1}H-^{13}C HMBC (heteronuclear multiple bond correlation) shows cross-peaks between protons and carbons that are two or three bonds away. With this experiment it is possible to study quaternary carbons that were not visible in the HSQC experiment. The HMBC experiment is used to establish the linkage between monosaccharide units via the glycosidic bond according to Figure 15.

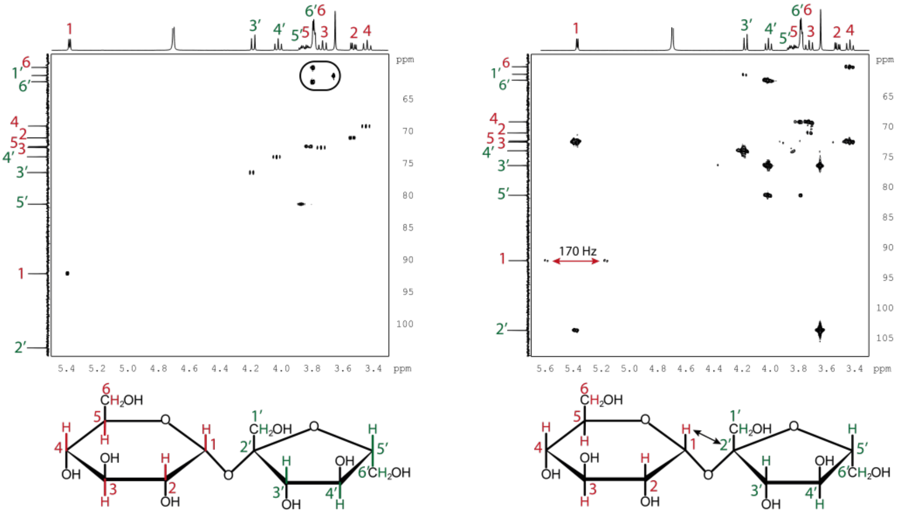


Figure 15. The HSQC spectrum of sucrose (left) shows the direct proton-carbon correlation through one bond coupling. The encircled CH/CH_3 signals will show the opposite sign to the CH_2 signals. The HMBC spectrum of sucrose (right) shows cross-peaks between protons and carbons two or three bonds away, and give also the possibility to measure the $^1J_{C1,H1}$ coupling constant as indicated in the figure.

Combination experiments

HSQC-TOCSY is a 2D TOCSY experiment that has an extra dispersion into the carbon dimension, which is especially useful when overlap of the different proton spin systems occur since carbon signals are usually better separated. From this experiment the assignments of individual spin systems in an oligosaccharide can be done. Cross-peaks are observed between all *J*-coupled protons and for all carbons in the spin system.

Through-space correlations

^{1}H-^{1}H NOESY (nuclear overhauser effect spectroscopy) correlations are arising from the spatial proximity between protons. The NOESY experiment can give information on how close two protons are in space, and NOEs are usually only observed between protons within monosaccharides or across the glycosidic linkage (Figure 16). Cross-peaks can be observed when the proton-proton distance is less than 5 Å. For small to medium sized molecules around 1 kDa, the NOE can be close to zero and no correlations are detected, this is because the strength of the interaction is dependent on the proton-proton distance, the external field and the correlation time. To overcome this problem, the temperature or the strength of the magnetic field can be changed or the ROESY experiment can be used instead (Figure 17).

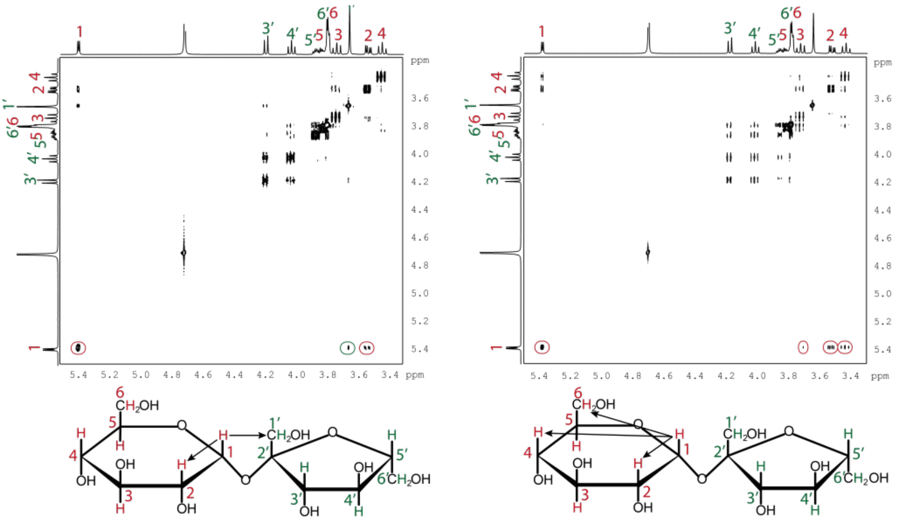


Figure 16. NOESY (left) and ROESY (right) spectra showing cross-peaks between protons with spatial proximity to each other.

^{1}H-^{1}H ROESY (rotating frame overhauser effect spectroscopy) correlation is also arising from spatial proximity between protons, but has the advantage of always being of positive sign and increases somewhat with molecular size (Figure 17).

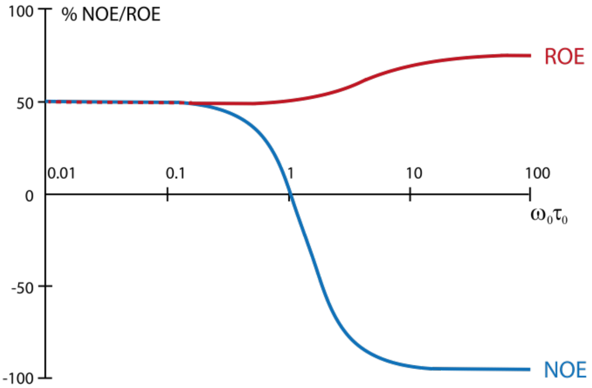


Figure 17. Schematic illustration of “the correlation time problem”, where a certain combination of field (ω_0) and molecular size (expressed as correlation time τ_0) can result in a zero or closed to zero NOE.

3.1.2 NMR of hydroxy protons

The large number of hydroxy protons in carbohydrates makes them valuable to study as they can give information about structure, hydrogen bonding and inter-proton distances (Sandström & Kenne, 2006; Poppe & Vanhalbeek, 1994; Symons *et al.*, 1980; Harvey *et al.*, 1976). One difficulty is that hydroxy protons are in a rapid exchange with protons of protic solvents at normal NMR conditions.

Sample preparation

Carbohydrates are usually studied by NMR using D_2O as the solvent. To be able to observe hydroxy protons, the solvent has to be changed from D_2O to H_2O (Figure 18), otherwise the protons will exchange with deuterons which are not visible in the ^1H NMR spectra. The use of H_2O as the solvent leads to the need of a water suppression experiment that does not affect the resonances of exchangeable protons. One efficient water suppression experiment that is often used is the WATERGATE experiment (Sklenar *et al.*, 1993; Piotto *et al.*, 1992). To reduce the exchange rate the temperature can be lowered to sub-zero temperatures and to avoid freezing 10-15 % acetone can be used. Further reduction of the exchange rate can be achieved by removal of impurities such as borate ions from the glassware and by adjusting the pH to around 5.5-7 (Adams & Lerner, 1992; Symons *et al.*, 1980; Harvey *et al.*, 1976). In the following section, the different NMR parameters that can be obtained from the hydroxy protons are discussed.

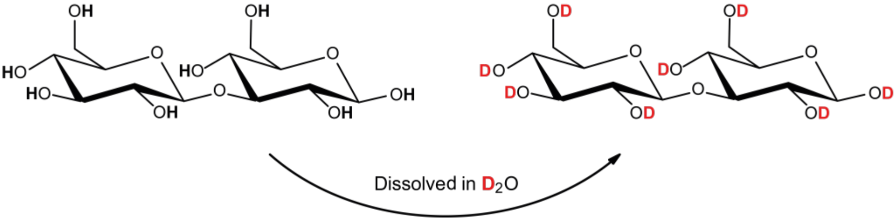


Figure 18. The exchange of the hydroxy protons for deuterons when a carbohydrate is dissolved in D_2O instead of H_2O.

Chemical shifts and chemical shift differences

Hydroxy protons in aqueous solution usually resonate in a well-isolated region of the NMR spectrum (δ 5-8 ppm). By comparing the chemical shift of a hydroxy proton in an oligosaccharide with that in the corresponding monosaccharide Δδ ($\Delta\delta = \delta_{oligo} - \delta_{mono}$) information on hydration and hydrogen bonding can be obtained.

The chemical shift of a hydroxy proton signal is a balance between two opposite contributions, a downfield shift due to hydrogen bonding interaction and an upfield shift due to reduced hydration (Bekiroglu *et al.*, 2004). An upfield shift (negative $\Delta\delta$) is an indication of reduced hydration due to steric hindrance or proximity to a ring oxygen. A downfield shift (positive $\Delta\delta$) is on the other hand an indication of spatial proximity to another hydroxyl group.

Vicinal $J_{CH,OH}$ *coupling constants*

According to a slightly modified Karplus equation derived for hydroxy protons in saccharides, a $^3J_{\mathrm{CH,OH}}$ value of around 5.7-5.8 Hz indicates a rotational averaging of the hydroxyl group around the C-O bond (Zhao *et al.*, 2007). A $^3J_{\mathrm{CH,OH}}$ value that significantly deviates from the rotationally averaged value could indicate that the hydroxyl group has a non-uniform distribution of rotamers due to steric reasons or hydrogen bonding.

Temperature coefficients (dδ/dT)

The temperature coefficient is calculated from the change in chemical shift of the hydroxy proton signal with temperature. It is a measurement of how much the hydroxy protons are interacting with the solvent through hydrogen bonding. Hydroxy protons that are fully hydrated and solely interact with the solvent have absolute temperature coefficient above 11 ppb/°C. Hydroxy protons with an absolute temperature coefficient below 5 ppb/°C are believed to be involved in strong intra-molecular hydrogen bond interactions (Kroon *et al.*, 1994; Poppe *et al.*, 1992).

NOEs and ROEs

NOESY and ROESY experiments can be used to study inter- and intra-molecular interaction involving hydroxy protons. In NOESY experiments it is not possible to distinguish between cross-peaks arising from cross-relaxation or chemical exchange contributions since they both have the same sign. In ROESY experiments, cross-peaks due to dipolar relaxation will have the opposite sign to the diagonal whereas cross-peaks due to chemical exchange will have the same sign as the diagonal peaks (Davis & Bax, 1985). Chemical exchange cross-peaks between hydroxy protons can be an indication of spatial proximity of hydroxyl groups and have been used to identify weak hydrogen bond interaction in for example sucrose (Sheng & Vanhalbeek, 1995), maltose (Bekiroglu *et al.*, 2003) and in hyaluronic acid oligosaccharides (Nestor *et al.*, 2010).

3.2 Mass spectrometry

Mass spectrometry (MS) is an important technique in structural elucidation of complex carbohydrates (Kailemia *et al.*, 2014). With the help of MS, information about the molecular mass, the identification and quantification of the constituent monosaccharides, the linkage sequence, branching point and stereochemistry of the carbohydrates can be obtained. There are three essential functions of a mass spectrometer; (1) ionization of the sample molecules, (2) separation of the ions according to their mass-to-charge ratio (*m/z*) and (3) detection of the ions. These functions are related to three different parts in the mass spectrometer, the ion source, the mass analyzer and the detector (Figure 19). There are several different types of ion sources and the type that is used depends on the volatility of the sample as well as on the nature of the sample. The ion sources most commonly used for analysis of carbohydrates are electron ionization (EI) for volatile samples, electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) for less volatile samples. These ionization techniques also differ in the received data, EI is a hard ionization technique and results in extensive fragmentation whereas ESI and MALDI are softer and can give information on the intact molecule. The mass analyzers used together with these ionization techniques are commonly quadrupole (Q) or time-of-flight (TOF) analyzers. The different types of detectors are recording the induced charge or the electric current that is produced when the ion hits the detector.

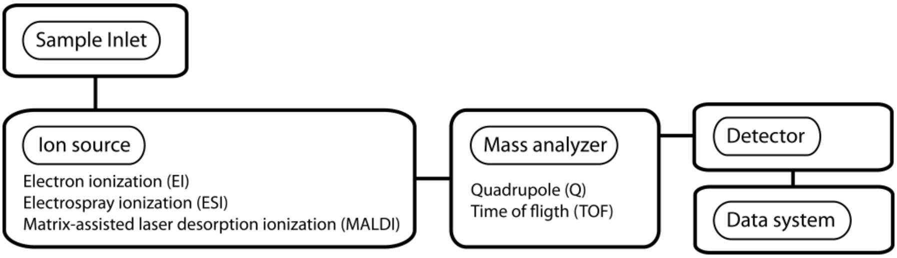


Figure 19. Schematic description of the different parts of a mass spectrometer.

3.2.1 Electron ionization

Electron ionization (EI) is a so called hard ionization technique that is used for volatile samples (Dempster, 1918). Sample molecules in the gas phase are bombarded with 70 eV electrons creating charged radical molecular ions, $M^{+\bullet}$, by the expelling of an electron. This process typically leads to extensive fragmentation and the molecular ions are not always observed. The extensive fragmentation can however be useful in elucidation of an unknown analyte. This ionization technique is only suited for molecules with a certain degree of volatility and is frequently used together with a gas chromatograph (GC) instrument. In characterization of the structure of carbohydrates, the identity of the constituent monosaccharides can be obtained through a monosaccharide constituent analysis (Sawardeker *et al.*, 1965). The oligo- or polysaccharide is hydrolyzed, reduced and acetylated before GC-MS or GC-FID analysis where the retention times of the monosaccharides are compared with authentic standards. It is possible through integration of the chromatographic peaks to measure the approximate relative amounts of the various monosaccharides. To study the position of the glycosidic linkage, a methylation analysis is performed. The oligo- or polysaccharide is methylated, reduced and acetylated before the GC-MS analysis (Ciucanu & Kerek, 1984). Specific fragmentation occurs since the C-C bond is preferably cleaved between two methoxy groups. The absolute configuration of carbohydrates can be studied by allowing the monosaccharide to react with (+) or (–) 2-butanol to give a 2-butyl glycoside, and by comparison with the retention time of prepared references (Gerwig *et al.*, 1978).

3.2.2 Electrospray ionization

Electrospray ionization (ESI) (Fenn *et al.*, 1990) is a soft ionization technique that can be used for non-volatile samples such as oligo- and polysaccharides. The ionization is obtained by spraying a solution of the sample at atmospheric pressure through a narrow capillary to which a high electric field is applied. The spray forms small charged droplets containing the analyte molecules. By exposing the droplets to heat and reduced pressure, the solvent is evaporated and the droplet size is reduced. As the coulomb repulsion exceeds the surface tension of the droplets they explode and gas phase ions of the sample molecules are formed. The ability to ionize compounds directly from an aqueous or aqueous/organic solution has made the ESI suitable to couple to an high-performance liquid chromatography (HPLC). The ESI soft ionization technique typically produces proton/sodium adducts or deprotonated molecular ions without fragmentation which makes it possible to observe the molecular ion, and from this the molecular weight can be determined. This makes it possible to deduce the composition of monosaccharides such as pentoses, hexoses, *etc*. but not to distinguish between different isobaric monosaccharides (Table 1).

Table 1. *Mass values for common monosaccharide residues (-H_2O).*

Monosaccharide	Examples	Formula	Monoisotopic mass	Average mass
Pentose	Arabinose (Ara), Ribose (Rib) Xylose (Xyl)	$C_5H_8O_4$	132.04	132.12
Deoxyhexose	Fucose (Fuc) Rhamnose (Rha)	$C_6H_{10}O_4$	146.06	146.14
Hexose	Glucose (Glc), Galactose (Gal) Mannose (Man)	$C_6H_{10}O_5$	162.06	162.14
N-Acetyl-aminohexose	*N*-Acetylglucosamine (GlcNAc) *N*-Acetylgalactosamine (GalNAc)	$C_8H_{13}NO_5$	203.08	203.19
Hexuronic-Acid	Mannuronic acid (ManA) Guluronic acid (GulA)	$C_6H_8O_6$	176.03	176.13
Sialic acid	*N*-Acetylneuraminic acid (NeuAc)	$C_{11}H_{19}NO_9$	291.09	291.26

In order to obtain structural information, tandem mass spectrometry (MS-MS) can be performed. In MS-MS, precursor ions in a narrow *m/z* region are isolated and allowed to collide with inert gas molecules. The collisions lead to the formation of fragment ions, which in turn are mass separated and detected. For carbohydrates, fragment ions are described using the nomenclature shown in Figure 20. The A, B, and C labels describe the fragment ions containing the non-reducing end, and the X, Y, and Z labels describe the fragment ions containing the reducing end (Domon & Costello, 1988).

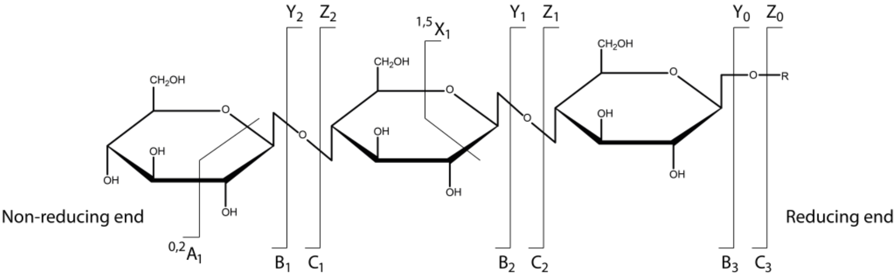


Figure 20. Different types of carbohydrate fragmentation described using the nomenclature by Domon and Costello.

3.2.3 Matrix-assisted laser desorption ionization

Matrix-assisted laser desorption ionization (MALDI) is also a soft ionization technique used for non-volatile compounds (Hillenkamp *et al.*, 1991). Here, the analyte molecules are dissolved in a solution of a UV-absorbing matrix compound. The analyte is allowed to co-crystallize with the matrix by placing a small amount of the sample-matrix solution onto a surface and let it to dry. Inside the MALDI ion source where vacuum is applied, the surface with the crystalline matrix-sample is exposed to pulses of UV laser light.

The ionization is achieved when the matrix absorbs energy from the laser, which is causing desorption of the matrix and analyte molecules into the gas phase. A fraction of the desorbed material is in the form of quasi-molecular ions e.g. $(M+H)^+$. The ions are transported into the mass analyzer via an electric field. The choice of matrix is important because different classes of molecules show large matrix dependent differences in ionization efficiency. The matrix should be an organic molecule that has absorption around the laser wavelength, has a low molecular mass, is vacuum stable, and promotes ionization. Examples of matrixes for carbohydrate analysis are 2,5-dihydroxybenzoic acid (DHB), α-cyano-4-hydroxycinnamic acid (CHCA) or trihydroxyacetophenone (THAP).

3.3 Chromatography

In the analysis of carbohydrates there is often a purification or separation step involved. Because of the large variety of isomeric or closely related structures, this requires high performance separation techniques. There are several separation techniques suitable for carbohydrates. Size-exclusion chromatography (SEC) separates molecules on the basis of molecular size (Churms, 1996b). Anion chromatography separates molecules based on their negative charge (Lee, 1996). Hydrophilic interaction liquid chromatography (HILIC) is a relatively new separation technique and the retention mechanisms are complex but are believed to be a mixture of partitioning, adsorption and ion exchange (Churms, 1996a). Porous graphite chromatography (PGC) separates molecules based on their hydrophobicity and planarity and this method has shown an extraordinary isomeric selectivity (Koizumi, 1996).

4 Result and Discussion

In this chapter, a summary of the results from papers I-IV are presented and discussed.

4.1 Paper I – NMR of Hydroxy protons of κ- and κ/μ-hybrid carrageenan oligosaccharides

The gel forming properties of κ-carrageenans are dependent on the ability of the polysaccharide to adopt a helical structure. The anhydro-bridge of the DA-residue allows formation of the helical tertiary structure, due to the $^{1}C_{4}$-conformation. The occurrence of the μ-precursor, lacking the anhydro bridge and with the $^{4}C_{1}$-conformation of the D residue (van de Velde *et al.*, 2002a), causes kinks in the regular helical structure of κ-carrageenan, and therefore affects the gelling ability. κ-Carrageenans are formed enzymatically *in vivo* from the precursor μ-carrageenans. *In vitro* the cyclization of the anhydro bridge is performed through alkali treatment.

It has been observed that the introduction of a small amount of the precursor ν-carrageenan in ι-carrageenans results in a decrease of helical formation while the rheological properties are remarkably enhanced (van de Velde *et al.*, 2002b). This is explained by the introduction of cross-links between the helical strands. It is the flexibility of the precursor that is essential for the formation of cross-linkages on the helical level. From an industrial point of view, the interest in the chemical fine structure and in the physical properties of carrageenans is due to the conversion of the precursors into the gelling polymers in the production process and how this process can be optimized to obtain the most suited properties for a specific usage (Hilliou *et al.*, 2006).

The structures of κ/μ hybrid carrageenan oligosaccharides produced from cultivated *Kappaphycus alvarezzi* by enzymatic digestion with *Pseudoalteromonas carrageenovora* κ-carrageenase were recently solved by NMR spectroscopy (Jouanneau *et al.*, 2010). In that study, a downfield shift of the anomeric (H1) proton of the internal D6S residue was observed when the μ-neocarrabiose unit was localized between two other μ-neocarrabiose moieties (Figure 21). Our hypothesis was that this downfield shift could originate from differences in hydrogen bonding interaction in the structures κμκ **5**, κμμκ **6** and κμμμκ **7**. To test this hypothesis and try to identify hydrogen bonding, a study of the hydroxy protons by NMR spectroscopy was performed. The oligosaccharides investigated are the κ and κ/μ-carrageenan oligosaccharides together with their constituent monosaccharides shown in Figure 21.

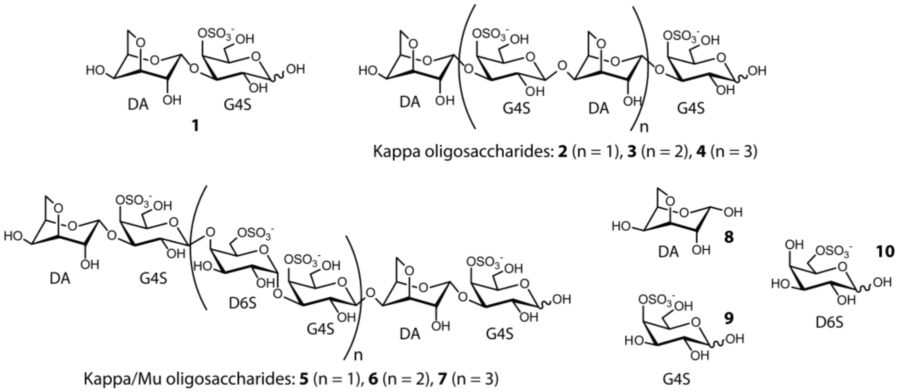


Figure 21. Structure of the studied oligosaccharides and the corresponding monosaccharides **1**-**10**.

Through the measurements of the chemical shifts (δ), chemical shift differences Δδ, temperature coefficients (dδ/dT) and chemical exchange of the hydroxy protons in κμμμκ **7**, it was suggested that the downfield shift of the anomeric proton on the D6S sugar of μ-neocarrabiose located between two other μ-neocarrabioses could be due to the loss of hydrogen bonding between OH3 of D6S (residue 6, from the reducing end) and OH2 of G4S (residue 7).

4.1.1 κ/μ hybrid carrageenan oligosaccharides

The assignment of the hydroxy protons was obtained from ^{1}H 1D and 2D COSY and TOCSY NMR spectra as well as with help from previously reported assignments of CH protons for several κ/μ-carrageenan oligosaccharides (Jouanneau *et al.*, 2010; Knutsen & Grasdalen, 1992). In the κμκ **5** and κμμκ **6**, the OH2_G4S signals with one or two neighboring D6S units (not the OH2_G4S(residue 3)) had very small Δδ. Comparison to the OH2_G4S with two DA units in κκ **3** and κκκ **4**, revealed a large downfield shift of the OH2_G4S signals with two neighboring D6S units (Figure 22). The corresponding OH2_G4S signals in κμμμκ **7** also had very small Δδ. No direct comparison could be made with the κκκκκ decasaccharide, but the OH2_G4S(5), (7), (9) had chemical shifts comparable to those observed for the octasaccharide κμμκ **6**.

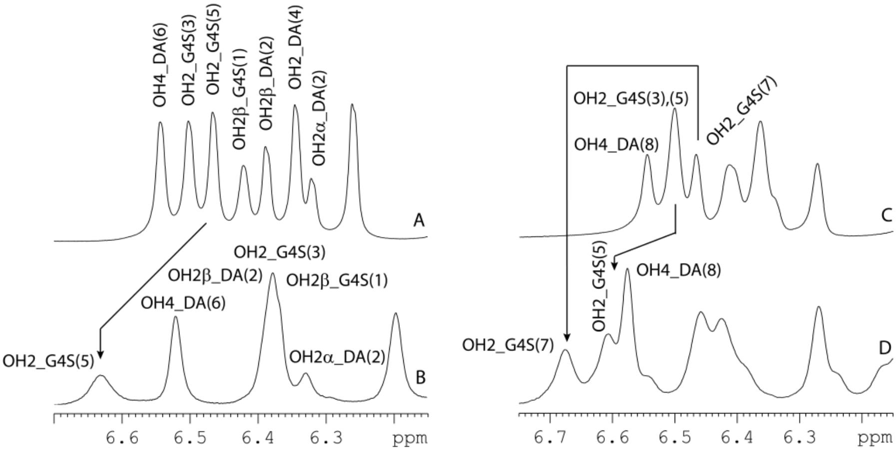


Figure 22. Portion of the ^{1}H NMR spectra showing the downfield shift of the OH2_G4S(5) signal in **5** (B) compared to in **3** (A), and of the downfield shift of the OH2_G4S(5) and OH2_G4S(7) signals in **6** (D) compared to in **4** (C).

OH2_D6S and OH3_D6S had temperature coefficients slightly lower than the other hydroxy protons. The lower |dδ/dT| values in structure **7** of approximately 2.0 ppb/°C compared to those in structures **5** and **6** is likely due to a pH difference between the samples. The NMR data for the hydroxy protons in the κ/μ-carrageenan oligosaccharides **5-7** are summarized in (Figure 23).

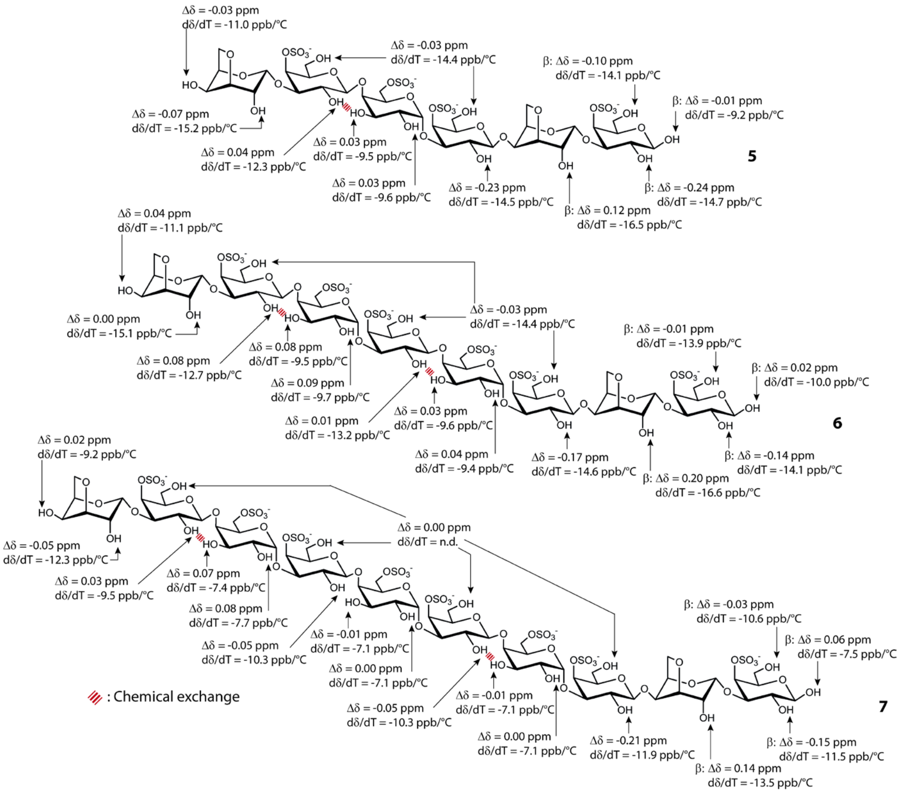


Figure 23. Δδ, dδ/dT, and chemical exchange of hydroxy protons on structures **5**-**7**. Only data for the β-anomeric form are shown (n.d. : not determined).

In κμκ **5** and κμμκ **6**, a chemical exchange interaction was observed in the ROESY spectrum between OH3_D6S(4) and OH2_G4S(5). In κμμκ, a second chemical exchange was observed between OH3_D6S(6) and OH2_G4S(7). For κμμμκ, **7,** two cross-peaks were observed in the TOCSY spectrum, one between OH3_D6S(8) and OH2_G4S(9) and one cross-peak including both OH3_D6S(4), OH2_G4S(5) and OH3_D6S(6), OH2_G4S(7) (Figure 24).

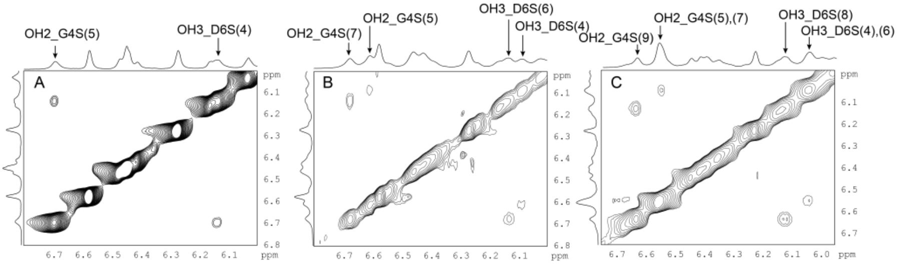


Figure 24. Part of TOCSY spectra showing the chemical exchange cross-peak between (A) in **5**, OH2_G4S(5) and OH3_D6S(4); (B) in **6**, OH2_G4S(5) and OH3_D6S(4); OH2_G4S(7) and OH3_D6S(6); (C) in **7**, the overlapping signals OH2_G4S(5), (7) and OH3_D6S(4), (6); OH2_G4S(9) and OH3_D6S(8).

The κμμμκ structure **7** has the possibility of forming three hydrogen bonds, between OH3_D6S(4) and OH2_G4S(5), OH3_D6S(6) and OH2_G4S(7) and between OH3_D6S(8) and OH2_G4S(9). However due to the overlapping of the OH3_D6S(4), (6) and OH2_G4S(5), (7) signals, it was not possible to unambiguously determine whether a chemical exchange also exists between OH3_D6S(6) and OH2_G4S(7), for the μ-carrabioside surrounded by two other μ-carrabiosides. Attempts to separate these overlapping signals by changing the pH, increasing the acetone-d_6 concentration and further decreasing the temperature were made, but sufficient separation was not achieved. Comparison of the cross-peak intensities in the TOCSY spectra of **6** and **7** suggested that only one of the pairs OH3_D6S(4), (6) and OH2_G4S(5), (7) had a chemical exchange interaction.

4.1.2 κ carrageenan oligosaccharides

For the κ-carrageenan oligosaccharides **1**-**4**, we did not observe any experimental evidence for occurrence of hydrogen bond interaction. The absolute value of Δδ was below 0.2 ppm for all hydroxy protons, which indicates that the hydration is similar to that in the constituent monosaccharides. Most of the hydroxy protons had high temperature coefficients |dδ/dT| in the range of 13-17 ppb/°C. Only the anomeric hydroxy protons OH1_G4S at the reducing end and OH4_DA of the non-reducing end showed slightly lower |dδ/dT| values. Only intra-residual NOEs/ROEs from the hydroxy protons to the ring protons were observed.

The slightly lower |dδ/dT| of OH4_DA at the non-reducing end was attributed to proximity to the anhydro bridge that restrict interaction with water while the negative Δδ of all OH2_G4S together with their slightly lower dδ/dT when compared to that of OH2_DA might be an indication of reduced hydration due to spatial proximity to the ring oxygen of DA.

A hydrogen bond between OH2_G4S and O5_DA has been found in the crystal of the neocarrabiose disaccharide and has also been predicted in several calculation studies (Bosco *et al.*, 2005; Stortz & Cerezo, 2003; Ueda *et al.*, 2001; Stortz & Cerezo, 2000; Lamba *et al.*, 1990). The NMR data for the hydroxy protons in **1-4** are summarized in Figure 25.

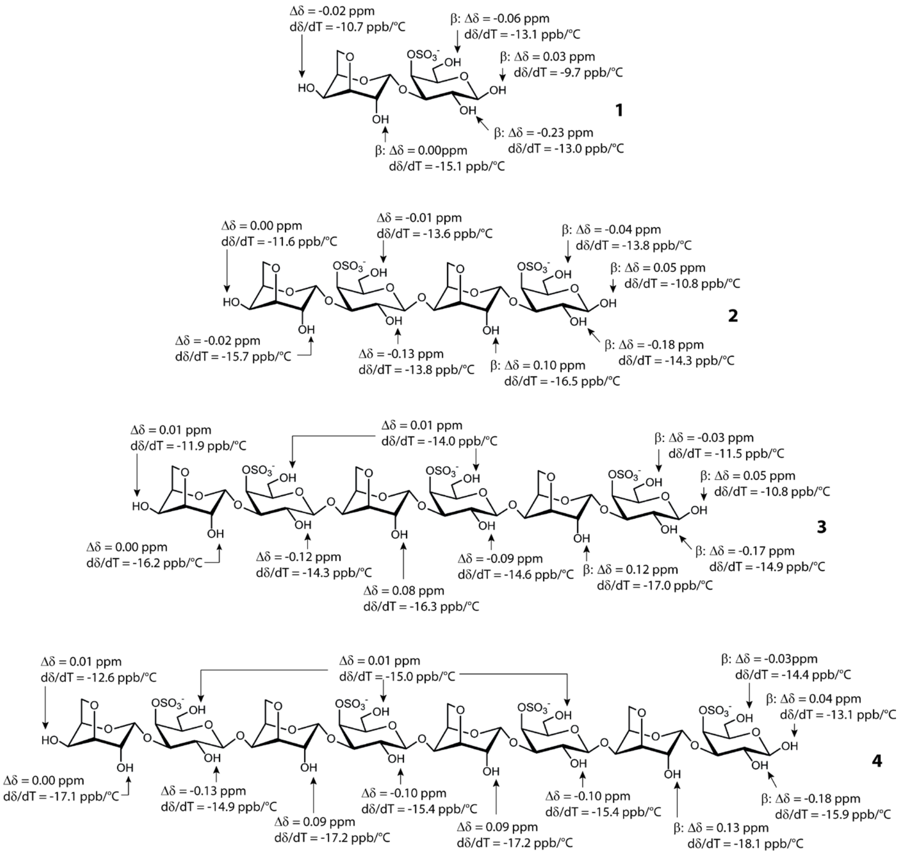


Figure 25. Δδ and dδ/dT of hydroxy protons on structures **1**-**4**. Only data for the β-anomeric form are shown.

4.1.3 Conclusion

The downfield shift of OH2_G4S in the κ/μ hybrid carrageenans in comparison to κ-carrageenans indicate a new chemical surrounding for OH2_G4S with a D6S residue as neighbor. OH2_G4S is close to another OH group and such interaction is not present in κ oligosaccharides. This, together with the chemical exchange between OH3_D6S and OH2_G4S suggest the existence of weak hydrogen bond interactions in the κ/μ hybrid carrageenans. The lower temperature coefficient of all OH2_D6S and OH3_D6S might reflect a reduced hydration resulting from spatial proximity to the neighboring 4-sulfate group of G4S through the α-glycosidic linkage and ring oxygen of G4S through the β-glycosidic linkage, respectively.

These interactions have been found from MM3 potential energy surfaces of trisaccharide model of μ-carrageenans (Stortz, 2006). For example, a hydrogen bond between OH2 of G4S and OH3 of D6S was predicted to stabilize the conformation of the β-glycosidic linkage. The present NMR data provide the first experimental evidence that such an interaction does exist, at least transiently, in κ/μ-oligosaccharides. A loss of a hydrogen bond in the decasaccharide for a μ-neocarrabiose when the neighbors are other μ-neocarrabioses could explain the downfield shift of the anomeric proton of the D6S sugar. However, the similarities in chemical shifts and temperature coefficients of OH2_G4S(5), (7) and OH3_D6S(4), (6), could also indicate similarities in the inner structure of the κμμμκ structure instead of structural differences.

4.2 Paper II and Paper III – Characterization of three alginate lyases from the two marine bacteria *Pseudomonas alginovora* and *Zobellia galactanivorans*

Alginate lyases can be used as sequencing tools to reveal the fine structure of alginates as well as to study the activity of C5-epimerases, hence there is a great interest of characterized alginate lyases. To develop such sequencing techniques, several alginate lyases with different specificity are required. Alginate lyases can also be used for the production of M- and G-blocks. In this work, one lyase from the marine bacteria *Pseudomaonas alginovora* and two lyases from the marine bacteria *Zobellia galactanivorans*, have been studied and characterized with respect to mode of action, substrate specificity, end products and minimal recognition pattern.

The cloning, expression and purification of these enzymes are described elsewhere; for the lyase from *P. alginovora* by Chavagnat *et al.* (1996), and for the two lyases, AlyA1 and AlyA5 from *Z. galactanivorans* by Thomas *et al.* (2012).

The optimal biochemical properties for enzymatic activity, such as optimal temperature, pH, salt concentration and effect of addition of divalent cations have been determined for all three enzymes. The procedure for the characterization of the alginate lyases, was to determine the substrate specificity by incubating the enzyme with different substrates, including crude alginate with M/G ratio of 0.9, M-blocks, G-blocks and alternating MG-blocks. The G-blocks, M-blocks and alternating MG-blocks were produced according to Haug *et al.* (1974). The end products obtained after hydrolysis of crude alginate were characterized by NMR and MS after separation and fractionation by SEC. The separation was monitored through the UV absorption at 230-235 nm, due to the conjugation between the double bond and the carboxylate group of the unsaturated Δ-residue (Figure 9). The minimal recognition pattern necessary for lyase activity was determined by allowing the enzymes to act on oligosaccharides of known size and structure.

4.2.1 Alginate lyase from *Pseudomonas alginovora*

The optimum temperature and salinity were 30 °C and 100 mM NaCl, and the enzyme showed a higher activity between pH 7 and 9, with a maximum at pH 9. The substrate specificity of the enzyme was determined by following the degradation of crude alginate, G-, M- and alternating MG-blocks directly in the NMR tube. Analysis of the NMR spectra before and after addition of enzyme demonstrated that the enzyme was acting only on M-M diads (Figure 26).

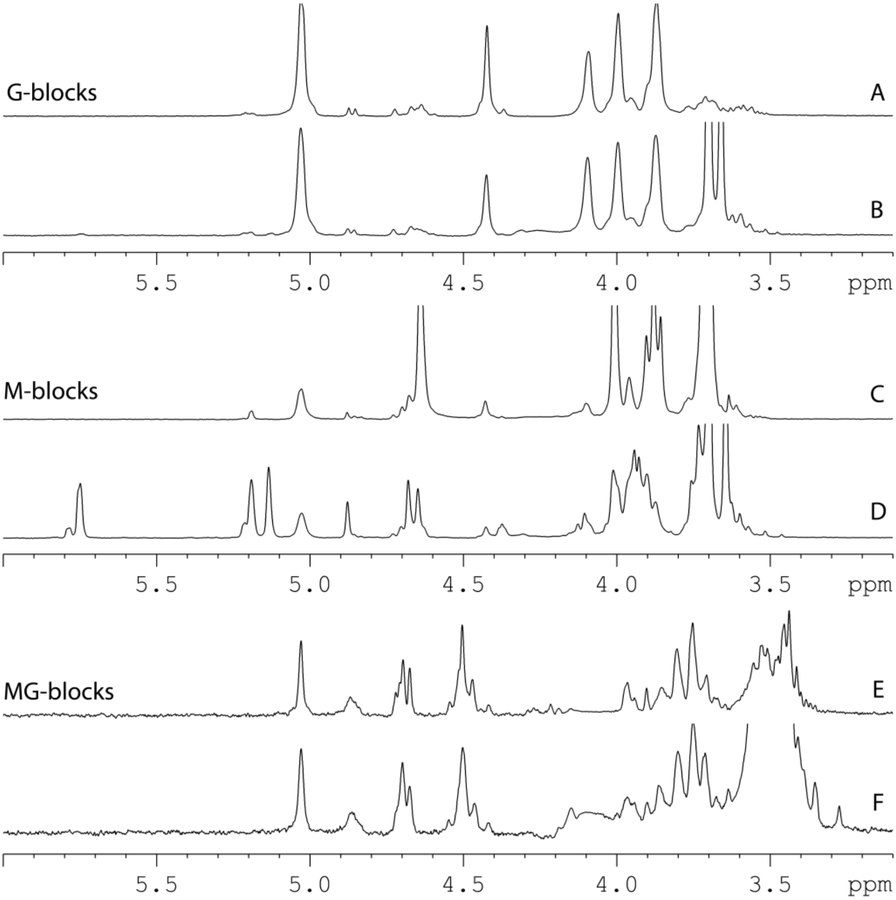


Figure 26. ^{1}H NMR spectra showing the effect of incubating G-, M- and MG-blocks with the alginate lyase from the marine bacteria *P. alginovora*. (A) G-blocks (B) G-blocks after incubation with the enzyme. (C) M-blocks (D) M-blocks after incubation with the enzyme. (E) MG-blocks (F) MG-blocks after incubation with the enzyme. No changes are observed in the NMR spectra of the G- and MG-blocks indicating that these structures are not substrate for the enzyme. For the M-blocks, incubation with the enzyme results in the apparition of signals at ~5.75, 5.20, 5.13 and 4.91 ppm characteristic for H4Δ(M), H1α(M), H1Δ(M) and H1β(M), respectively, and showing that the enzyme is cleaving between two M residues.

The separation profile (Figure 27) obtained from the SEC showed four major peaks from which the molar fraction of the unsaturated oligosaccharides was estimated.

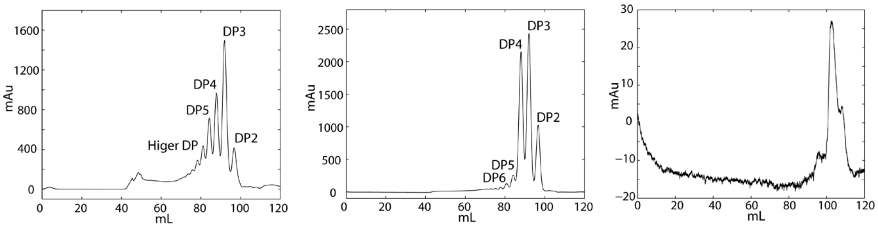


Figure 27. SEC profile of unsaturated oligosaccharides produced by incubation of crude alginate with alginate lyase; (left) from *P. alginovora*, (middle) AlyA1 from *Z. galactanivorans*, and (right) AlyA5 from *Z. galactanivorans*.

The degree of polymerization of the fractions was determined by ESI-MS analysis. The lyase was found to produce mainly trisaccharide and tetrasaccharide oligomers with a total content of 49 and 21 %, respectively. Around 12 % of disaccharide and pentasaccharide oligosaccharides were isolated. NMR analysis of these four isolated fractions showed that they contained only ΔM, ΔMM, ΔMMM and ΔMMMM structures, respectively. The minimal recognition oligosaccharide was concluded to be the hexasaccharide MMMMMM, due to the high content of unsaturated di-, tri- tetra- and pentasaccharide isolated together with the fact that the pentasaccharide MMMMM was not cleaved by the enzyme.

The amino acid sequence of the *P. alginovora* alginate lyase was used with the BLAST algorithm to find information about the enzyme class, but no significant similarities were found between this enzyme and any known alginate lyase. Thus, even if this alginate lyase preferentially cleave poly(M) substrate with an *endo*-mechanism, as most of the alginate lyases investigated so far, it appears that it has evolved through a different evolutionary pathway, leading to an alginate lyase activity by convergent evolution.

4.2.2 Alginate lyase AlyA1 from *Zobellia galactanivorans*

The optimum temperature and salinity were 30 °C and 200 mM NaCl, respectively, and the highest activity of the enzyme was obtained at pH 7 in Tris-HCl buffer. The substrate specificity for AlyA1 was determined by following the degradation of crude alginate, G-, M- and alternating MG-blocks directly in the NMR tube, and it was observed that the enzyme was active only on G-G diads.

The oligosaccharides produced from degradation of the crude alginate were separated and fractionated by SEC, and the separation profile (Figure 27) showed three major peaks from which the molar fraction of the unsaturated oligosaccharides was estimated. The degree of polymerization of these fractions was determined by ESI-MS analysis. AlyA1 produced mainly trisaccharide and tetrasaccharide oligomers with a total content of 41 and 36 % respectively. Around 19 % of disaccharide, and only a small amount of penta- and hexasaccharide oligosaccharides were isolated. The NMR spectra of the fractions containing the di- and trisaccharides revealed that ΔG was the only disaccharide produced whereas two trisaccharides, ΔGG and ΔMG, were present in the amounts of 88 and 12 % respectively. The NMR spectra of the tetrasaccharide fraction showed the presence of several structures. In the ΔH4 region, three signals were assigned to ΔGMX, ΔGGX, and ΔMXX (with X being a M or G residue), according to the chemical shift values at 5.75, 5.74, and 5.67 ppm respectively (Figure 28).

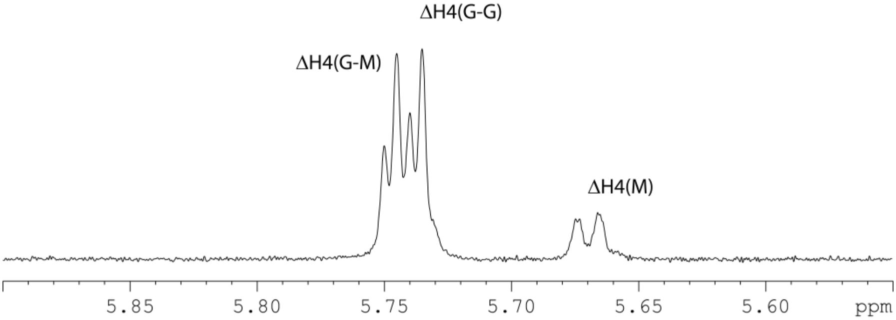


Figure 28. Expansion of the ΔH4 region of the ^{1}H NMR spectrum of the tetrasaccharide fraction.

In the region of the reducing end H2(G) (Figure 29), two well separated signals with different neighbors were observed, with the characteristic chemical shift at 3.44 ppm for the reducing end with a G neighbor and at 3.51 ppm for the reducing end with a M neighbor. Since the enzyme produces only compounds with G-reducing end sugar, it was concluded that the following structures were present; ΔGMG, ΔGGG, and ΔMXG. The structure of ΔMXG was determined using selective TOCSY experiments. Two ΔH2(M) signals were observed in the NMR spectra (Figure 29). Thus, four different structures were present in the tetrasaccharide fraction; ΔGGG, ΔGMG, ΔMGG, and ΔMMG. Integration of the H4ΔG and H4ΔM signals indicated that ΔGGG and ΔGMG together represent 84 % of the total content of tetrasaccharides, whereas ΔMGG and ΔMMG account for 16 % of the molecules.

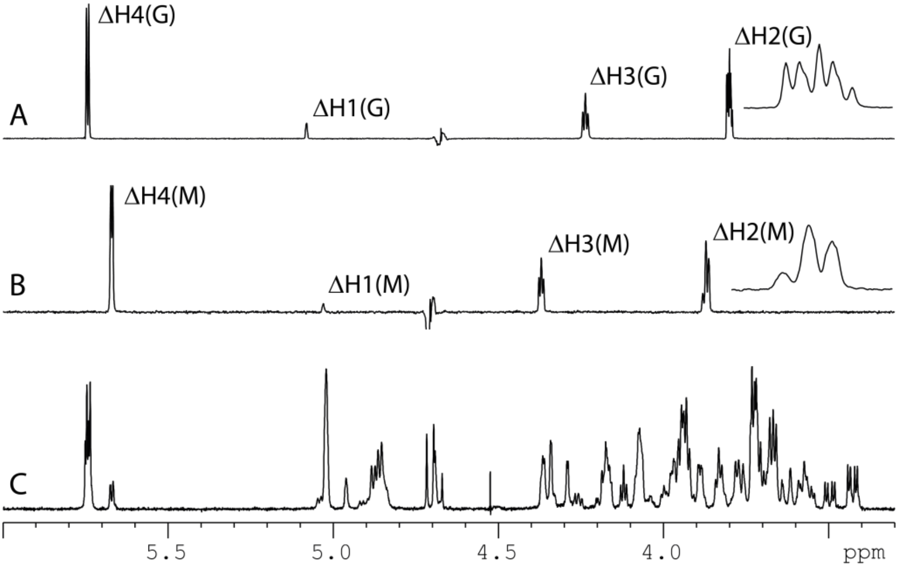


Figure 29. Selective TOCSY of the tetrasaccharide products produced by AlyA1. (A) selective excitation of ΔH4(G), (B) selective excitation of ΔH4(M), and (C) ^{1}H NMR spectrum of the tetrasaccharide.

Since analysis of the end products revealed a high content of di-, tri- and tetrasaccharides and low content of penta- and hexasaccharides, it was concluded that the minimal recognition oligosaccharide is a pentasaccharide. Further NMR experiments showed that the hexasaccharide GGGGGG was degraded by AlyA1. Purified pentasaccharides were not available but the structures degraded by the enzyme were deduced from MNR analysis of the end products. These pentasaccharides are: GGGGG, GGMGG, GGGMG, and GGMMG.

4.2.3 Alginate lyase AlyA5 from *Zobellia galactanivorans*

The optimum temperature was 30 °C and the enzyme showed highest activity at pH 7. The salinity was held at 100 mM NaCl as for AlyA1. The substrate specificity of AlyA5 was determined by following the degradation of G-, M- and alternating MG-blocks directly in the NMR tube. All three block types were degraded by the enzyme. In the NMR spectra, beside the characteristic ΔH4 signals indicating the formation of unsaturated oligosaccharides, additional signals were observed in two regions, 1.6-2.6 and 3-4 ppm (Figure 30).

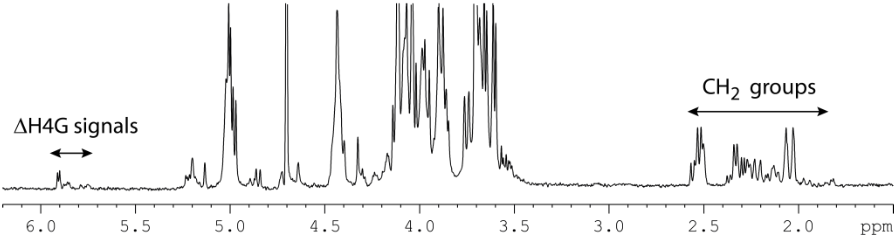


Figure 30. ^{1}H NMR spectrum showing the signals from CH_2 groups arising from the spontaneous conversion of Δ into DEH.

The end products of the AlyA5 reaction were characterized by following the degradation of G-blocks. During the course of the reaction, the concentration of the disaccharide ΔG was found to increase although the concentration of the longer unsaturated oligosaccharides decreased. A small amount of the unsaturated monosaccharide Δ (4-deoxy-L-*erythro*-hex-4-ene pyranosyluronat) was identified even though it was unstable and spontaneously converted into DEH (4-deoxy-L-*erythro*-5-hexoseulose uronic acid) (Figure 10). The signals observed between 1.6-2.6 and 3-4 ppm correspond to protons from CH_2 groups in DEH (Figure 30). The concentration of ΔG was always low during the course of the reaction, when compared with DEH, showing that ΔG is a minor degradation product.

The minimal recognition oligosaccharide of AlyA5 was studied by incubation of the enzyme with saturated, and unsaturated trisaccharides (Figure 31). The saturated MMM and GGG were degraded by the enzyme producing ΔM and ΔG, respectively. ΔGG was degraded into Δ and ΔG. ΔMG was also producing Δ and ΔG, no ΔM was observed indicating that AlyA5 is able to cleave the glycosidic linkage between the Δ and M sugar but also that it is degrading the substrates from the non-reducing end. AlyA5 also degraded the unsaturated ΔMM (ΔGM was not available). Disaccharides were not substrates for the enzyme revealing that the minimal recognition oligosaccharides are saturated and unsaturated trisaccharides.

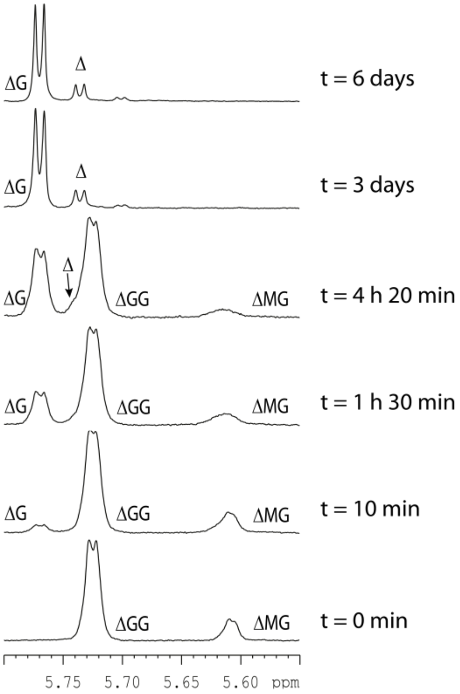


Figure 31. Portion of the proton NMR spectra showing the ΔH4 signals upon degradation of ΔGG and ΔMG by AlyA5 as a function of time. Both ΔGG and ΔMG are degraded to Δ and ΔG.

4.2.4 Conclusion

Three new and complementary alginate lyases have been characterized. The alginate lyase from *Pseudomonas alginovora* has an endolytic activity with a strict specificity for M-block alginates.

AlyA1 from *Zobellia galactanivorans* showed an endolytic activity with a strict specificity for G-block alginates. The crystal structure of AlyA1 showed an active site with an open cleft architecture (Figure 32), which is common for endolytic enzymes. An open cleft can accommodate longer sugar chains with four or more monosaccharide units.

These two alginate lyases from *P. alginovora* and *Z. galactanivorans* can be used as tools for sequencing of alginates or in the preparation of G- and M-blocks of alginates, respectively.

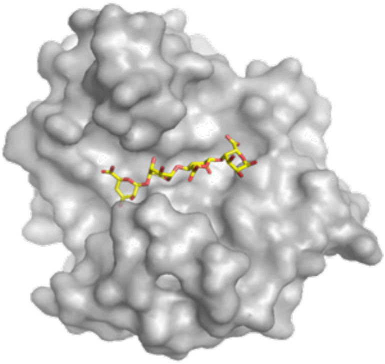


Figure 32. Surface representation of the crystal structure of AlyA1. A tetrasaccharide GGGG is superimposed, showing the open cleft.

The AlyA5 alginate lyase from the marine bacteria *Z. galactanivorans* has the more unusual exolytic activity with a broad substrate tolerance. The ability to degrade oligosaccharides down to mono- and disaccharides has only been seen for a few alginate lyases before (Kim *et al.*, 2012; Hashimoto *et al.*, 2000). The crystal structure of AlyA5 showed an active site of pocket architecture (Figure 33), which is typical for exolytic enzymes. This type of short pocket binding site are known for recognizing short sugar ligands containing one to three monosaccharide units, and acts from the reducing or non-reducing ends.

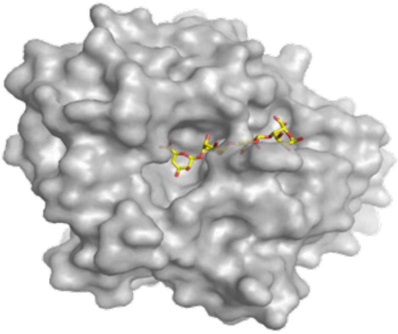


Figure 33. Surface representation of the crystal structure of AlyA5. A tetrasaccharide GGGG is superimposed, showing the pocket architecture.

AlyA1 and AlyA5 are together able to degrade alginate polymers down to monosaccharide (Figure 34).

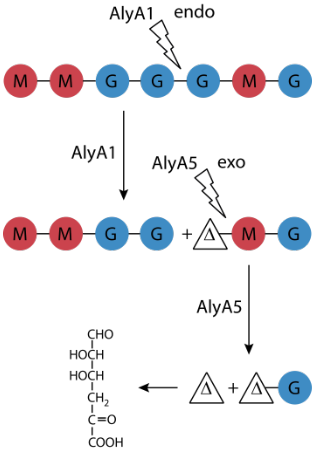


Figure 34. Schematic representation of the mode of action of the two complementary alginate lyases AlyA1 and AlyA5 from the marine bacteria *Z. galactanivorans*.

4.3 Paper IV – Structural study of the LPS from the Gram-negative bacteria *Plesiomonas shigelloides*

The LPS was extracted from bacterial cells of *Plesiomonas shigelloides* by the hot phenol/water method (Westphal & Jann, 1965). The LPS recovered from the water phase was dialyzed and ultra-centrifugated. The LPS was delipidated with acetic acid and fractionated by SEC, yielding three main fractions, PS, OSI and OSII. Analysis by NMR and ESI-MS showed that the PS fraction consisted of the core oligosaccharide substituted with the O-specific polysaccharide whereas the OSI fraction consisted of the core with one repeating unit and the OSII fraction of the unsubstituted core.

4.3.1 The O-specific repeating unit

The O-specific polysaccharide was found to contain the following tetrasaccharide repeating unit (Figure 35).

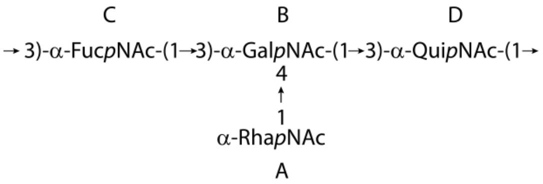


Figure 35. O-specific repeating unit of the LPS from *P. Shigelloides* O24:H8.

In the HSQC spectrum, four anomeric proton resonances at 5.19, 5.13, 5.11 and 4.93 ppm showed correlation to anomeric carbon resonances at 98.50, 97.92, 95.77 and 99.09 ppm, respectively, indicating the presence of four sugar residues in the repeating unit (Figure 36). The signals were annotated with capital letters A-D according to decreasing chemical shift values. In the ^{1}H NMR spectrum, three upfield shifted resonances were observed, two at 1.23 ppm and one at 1.26 ppm, characteristic for methyl groups of 6-deoxy sugars. Three signals at 2.02 and 2.04 ppm together with one with twice the intensity at 1.95 ppm, indicated four methyl groups from the *N*-acetyl function in acetamido sugars. The HSQC spectrum showed four resonances in the range 47-55 ppm characteristic for carbons bound to nitrogen, which is consistent with the four acetamido groups in the repeating unit. The downfield shift of the anomeric protons, together with the $^{1}J_{C1,H1}$ values (172, 174, 175 and 179 Hz) indicated the α-pyranosyl configuration for all four residues. The assignments of residues A-D were obtained from COSY, TOCSY, NOESY and HSQC-TOCSY experiments.

The anomeric resonance at 5.18 ppm, residue A, showed a weak cross-peak to H2 at 4.37 ppm, H2 showed a cross-peak to H3 at 3.97 ppm and from H3 a cross-peak to H4 at 3.36 ppm was observed in the COSY spectrum. The weak cross-peaks in the COSY spectrum indicate small $^{3}J_{H1,H2}$ and $^{3}J_{H2,H3}$ coupling constants. Through a TOCSY experiment the connectivities from H4 to H5 at 3.67 ppm and H6 (methyl) at 1.23 ppm were observed. The H4 signal was well resolved and appeared as a triplet which indicates large and similar $^{3}J_{H3,H4}$ and $^{3}J_{H4,H5}$ coupling constant values. In the HSQC spectrum, the H2 proton showed correlation with the carbon resonance at 54.05 ppm (Figure 36) indicating an *N*-acetyl function at the C2 position. In the NOESY spectrum a strong cross-peak between H2 and H3 was observed. From these data, residue A was assigned as a 2-acetamido-2,6-dideoxy-hexo pyranose with a *manno*-configuration (RhaNAc). The α-anomeric configuration was further confirmed by the cross-peaks between H1 and H2 and H2 and H3 in the NOESY spectrum, together with the absence of cross-peaks between H1 and H3 as well as between H1 and H5 expected for the β-pyranosyl configuration.

The anomeric resonance at 5.13 ppm, residue B, showed a cross-peak to H2 at 4.60 ppm, H2 showed a cross-peak to H3 at 3.98 ppm, and H3 showed a cross-peak to H4 at 4.59 ppm in the COSY spectrum. H1 showed NOE to H2, H3 showed NOE to H5 at 4.87 ppm and H5 showed NOE to H4. These data indicated a residue with *galacto*-configuration. In the HSQC spectrum, H2 showed a correlation with the carbon resonance at 48.8 ppm (Figure 36) indicating an *N*-acetyl function at the C2 position.

The absence of an H6 resonance and the downfield shift of H4 and H5 (Figure 36) are characteristic for an uronic acid and residue B was thus identified as a 2-acetamido-2-deoxy-galacturonic acid (GalNAcA).

The anomeric resonance at 5.11 ppm, residue C, showed a cross-peak to H2 at 4.37 ppm, and H2 showed a cross-peak to H3 at 3.65 ppm in the COSY spectrum. NOESY showed cross-peaks between H1 and H2, as well as between H3 and H4, H3 and H5 and H4 and H5. In the TOCSY spectrum, H5 at 3.79 ppm showed connectivity to H6 (methyl) at 1.26 ppm. In the HSQC spectrum, H2 correlated with the carbon resonance at 49.4 ppm (Figure 36) indicating an *N*-acetyl function at the C2 position. Residue C was assigned to be a 2-acetamido-2,6-dideoxy-hexo pyranose with *galacto*-configuration (FucNAc)

The anomeric resonance at 4.93 ppm, residue D, gave a cross-peak to H2 at 4.14 ppm in the COSY spectrum. In the TOCSY spectrum, correlations from H1 to H6 (1.26 ppm) were observed. Both H3 and H4 signals appeared as a triplet indicating similar values of $^3J_{H2,H3}$, $^3J_{H3,H4}$ and $^3J_{H4,H5}$ coupling constants. In the HSQC spectrum, H2 correlated with the carbon resonance at 54.4 ppm (Figure 36) indicating an *N*-acetyl function at the C2 position. The α-anomeric configuration was further confirmed from the strong cross-peaks observed between H1 and H2 in the NOESY spectrum. From these results, residue D was concluded to be an α-2-acetamido-2,6-dideoxy-hexo pyranose with *gluco*-configuration (QuiNAc).

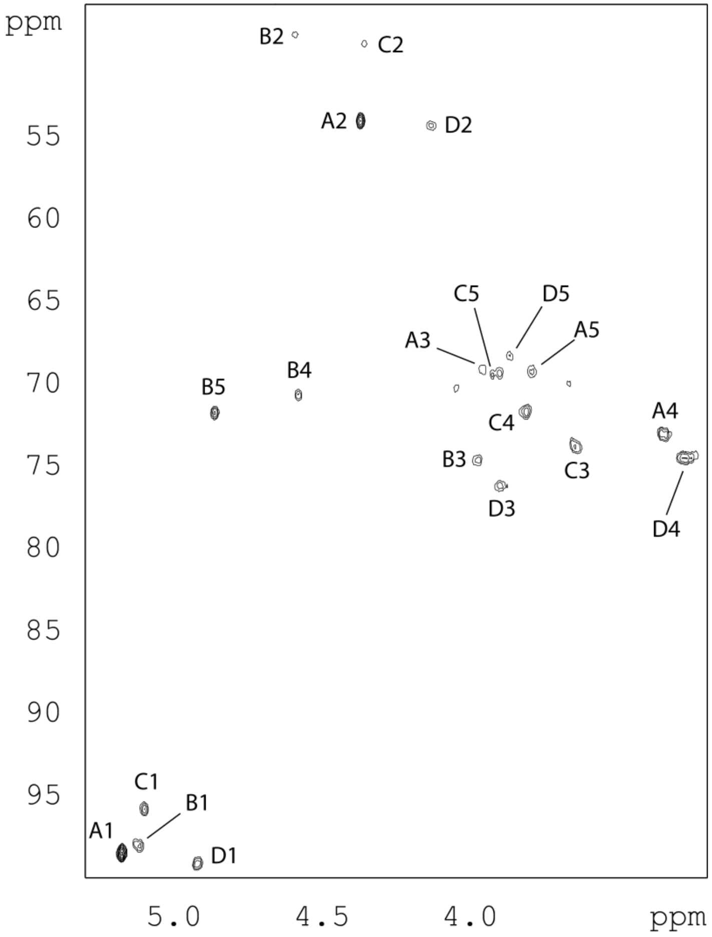


Figure 36. HSQC spectrum of the O-specific repeating unit of the LPS from *P. Shigelloides* O24:H8.

The sequence of the monosaccharide residues and the connections between them were obtained from the inter-residue interactions observed in NOESY and HMBC spectra. Inter-residues NOEs were found between H1(A) and H4(B), H1(B) and H3(D), H1(D) and H3(C), H1(C) and H3(B) (Figure 37). This sequence was confirmed by HMBC cross-peaks between the anomeric protons and the carbons at the linkage position.

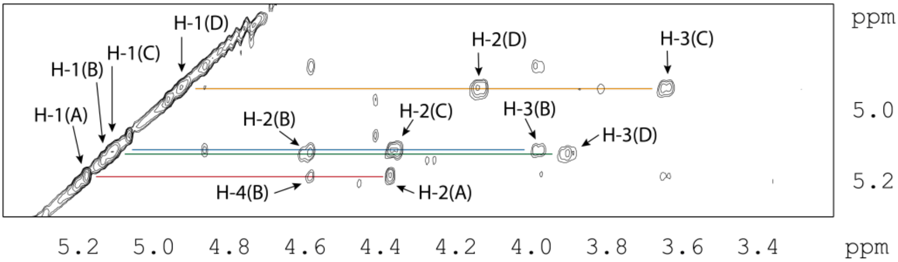


Figure 37. Portion of the NOESY spectrum of the O-specific repeating unit of the LPS from *P. Shigelloides* O24:H8.

The mass of the O-specific repeating unit (779.4 Da) was determined from ESI-MS by comparing the mass of the OSI fraction with the mass of the OSII fraction and was in good agreement with the structure obtained by NMR (Figures 38-39).

4.3.2 The core oligosaccharide

From a serological screening of 69 different O-serotypes of *Plesiomonas shigelloides*, Niedziela *et al.* (2002) suggested that epitopes similar to the core oligosaccharide of the serotype O54 (strain CNCTC 113/92) could also be present in the core region of the serotype O24 (strain CNCTC 92/89).

The ESI-MS analysis of the OSII fraction showed two main ions at *m/z* 821.8 $[M\text{-}H_2O+H+Na]^{2+}$ and *m/z* 902.8 $[M\text{-}H_2O+H+Na]^{2+}$, giving the monoisotopic mass of 1637.6 Da and 1799.6 Da respectively, differing only in one hexose unit (162 Da) and suggesting a decasaccharide and a nonasaccharide respectively (Figure 39). The nine sugars present in *P. shigelloides* CNCTC 113/92, two Gal, one Glc, three Hep, one GalA, one GlcN and one Kdo, gave together a monoisotopic mass of 1637.5 Da, which is in agreement with one of the masses of OSII.

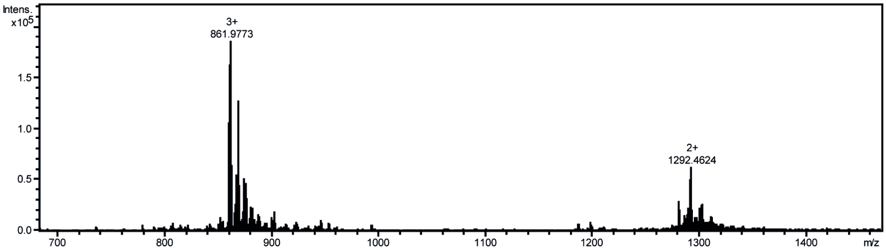


Figure 38. Mass spectrum of the OSI fraction containing the core oligosaccharide substituted by one repeating unit.

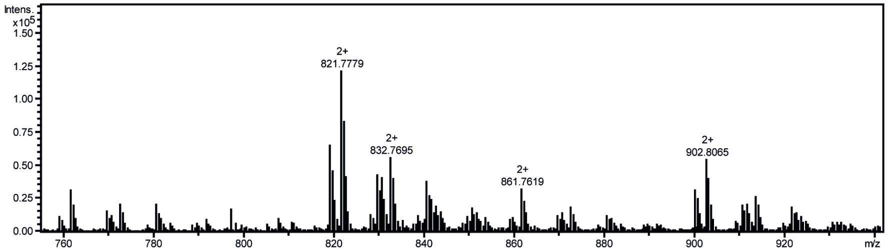


Figure 39. Mass spectrum of the OSII fraction containing the unsubstituted core oligosaccharide.

The HSQC spectrum (Figure 40) of the OSII fraction showed eight main signals in the region for anomeric protons and carbons at 5.45/101.1 ppm (**E**), 5.32/99.9 ppm (**F**), 5.21/96.50 ppm (**G**), 5.12/100.9 ppm (**H**), 4.93/100.9 ppm (**I**), 4.59/103.4 ppm (**J**), 4.47/103.7 ppm (**K**), and 4.43/104.1 ppm (**L**), as well as a spin system corresponding to a Kdo (**M**) (not shown in Figure 40) residue indicating a nonasaccharide core structure.

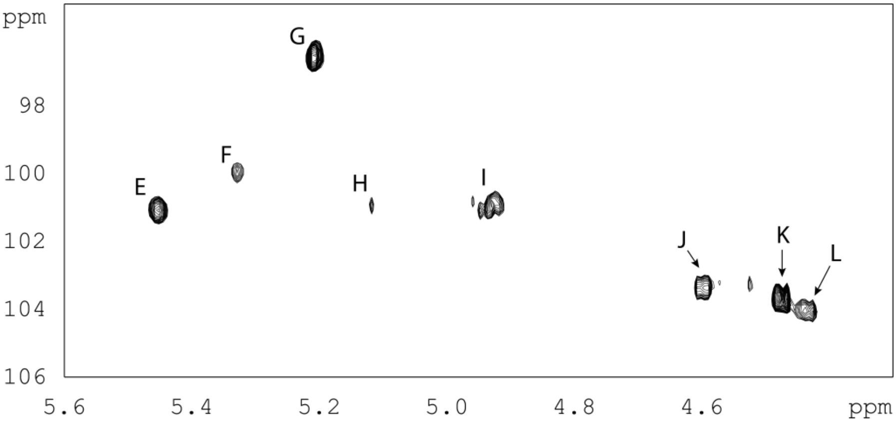


Figure 40. Anomeric region of the HSQC spectrum of the core oligosaccharide of the LPS from *P. Shigelloides* O24:H8.

NMR analysis of the OSI fraction showed an additional signal in the anomeric region at 4.47/103.6 ppm (**N**), corresponding to a sugar residue with *gluco*-configuration. This residue was shown to be 1→6 linked to α-D-Glc*p*N (**G**) from the cross-peak between H1 (**N**) and H6 (**G**) at 4.16 ppm in the NOESY spectrum, and from the cross-peak between H1 (**N**) and C6 (**G**) at 67.9 ppm in the HMBC spectrum. Comparison of the NMR data of the core oligosaccharide with those published for the core of the LPS from *P. shigelloides* CNCTC 113/92 (Niedziela *et al.*, 2002), showed that the two structures were similar (Figure 41).

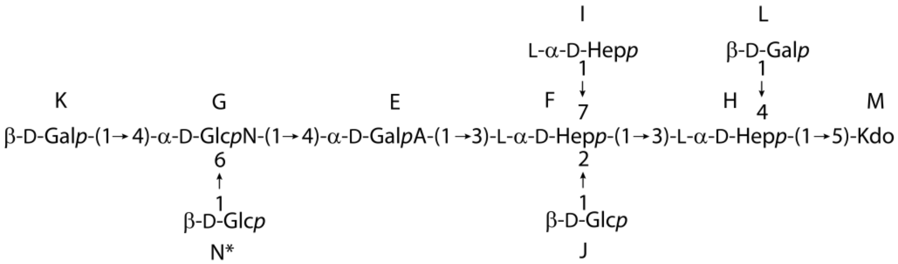


Figure 41. Structure of the core oligosaccharide of the LPS from *P. shigelloides* O24:H8 (Strain CNCTC 92/89).

4.3.3 Conclusion

A structure similar to that of the tetrasaccharide repeating unit has been reported for the capsular polysaccharide of the *Vibrio vulnificus* Strain BO62316 (Reddy *et al.*, 1998; Reddy *et al.*, 1993). The structural similarities might be explained by the fact that *Plesiomonas shigelloides* belonged to the *Vibrionaceae* family before it was moved to the *Enterobacteriaceae* family. Another strain of *P. shigelloides* serotype O17 has shown to express an O-specific polysaccharide identical to the *Shigella sonni* O-specific polysaccharide (Kubler-Kielb *et al.*, 2008).

It has been suggested that the presence of deoxy sugars with hydrophobic substituents in all the O-specific polysaccharides structures so far characterized rendering a high hydrophobic LPS could suggest a method adopted by *P. shigelloides* to adhere to host cells in aqueous environment (Aquilini *et al.*, 2013; Nazarenko *et al.*, 2011; Maciejewska *et al.*, 2009).

5 Conclusions and Further Perspectives

κ-Carrageenans give strong gels while κ/μ-carrageenan hybrids have low gelling properties. The structural implications of μ-residues in κ-carrageenan oligosaccharides were investigated using NMR of hydroxy protons. The κ, κκ, κκκ, κκκκ, κκκκκ, κμκ, κμμκ and κμμμκ oligosaccharides were analyzed. A weak hydrogen bonding interaction was found across the 1→4 glycosidic linkages of μ-carrabiose in the κμκ, κμμκ and κμμμκ oligosaccharides. In κμμμκ the NMR data suggested that this hydrogen bond might be lost in the central μ-carrabiose unit.

Gelation is based on intermolecular hydrogen bonds in large polymers while the NMR data presented here only show intramolecular hydrogen bonds in oligosaccharides. It cannot be excluded however that what we observed at the oligosaccharide level is also present in the larger polymers. Studies of larger κ/μ-hybrid structures that can form helical strands could give new information important for describing the structural implications of hybridity in carrageenans.

Three new alginate lyases, the endolytic M-lyase from *Pseudomonas alginovora*, the endolytic G-lyase, AlyA1, from *Zobellia galactanivorans* both acting on alginate polymers and the exolytic AlyA5 active on oligosaccharides and with a broad substrate tolerance, were characterized. These enzymes, with complementary functions and known mode of action, could be employed to develop a fingerprinting method for sequencing of alginates using for example LC-MS.

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