Studies of Carbohydrate Structure, Properties and Interactions by NMR Spectroscopy

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Cover: 3D representation of a κ/μ-hybrid-carrageenan hexasaccharide superimposed on a part of the TOCSY spectrum of the hexasaccharide. The crosspeaks represent the chemical exchange visualized in the figure by the centremost dashed blue line. The other two dashed blue lines represent transient hydrogen bonding interactions.

(Image: Eric Morssing Vilén)

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Abstract

In this thesis the structure, properties and interactions of different types of carbohydrates were investigated by NMR spectroscopy.

The structures of kappa- (κ) and kappa/mu- (κ/μ) carrageenan oligosaccharides were analyzed by studying the hydroxy protons. It was shown that a hydrogen bonding interaction is present across the 1→4 glycosidic linkages of μ-carrabiose in the κμκ hexa- and κμμκ octasaccharides. The occurrence of hydrogen bonding in κ/μ-carrageenan oligosaccharides may suggest that μ-carrabiose units, mostly found in the non-helicoidal regions of κ-carrageenans have an underestimated role in the structural organization of the κ-carrageenan gel network.

Hydroxy proton NMR was also used to study the effect of trehalose on the hydration and hydrogen bonding in lactose in aqueous solutions. The small effects of trehalose on the hydration and hydrogen bonding interaction in lactose were very similar to those found for sucrose. The results suggested that, at concentrations below 40% (w/w), it is the concentration of hydroxy groups that governs sugar-sugar and sugar-water interactions rather than the type of sugar.

A method using diffusion-edited NMR spectroscopy was developed for solvent suppression when determining the mannuronic (M) to guluronic (M) acid ratio in alginate polysaccharides. The method could be employed to determine the M/G-ratio at temperatures below 50 °C. Through all of the work in the thesis diffusion-edited NMR experiments also proved to be practical for studies of biomolecules, to for example selectively remove interfering signals from buffer to enable the interpretation of sample signals.

The activities and specificities of four different glycosaminoglycan (GAG) sulfatases from the human gut commensal *Bacteroides thetaiotaomicron* were determined. One of the sulfatases, BT3349, was found to be the first bacterial GAG endolytic-*O*-sulfatase, with chondroitin specific GalNAc-4-*O*-sulfatase activity. The other three enzymes were shown to possess strictly exolytic activity, BT3333 as a GalNAc-6-*O*-sulfatase, BT4656 as a GlcNAc-6-*O*-sulfatase and the third one, BT1596, as a Δ-4-hexuronate-2-*O*-sulfatase.

Keywords: NMR, carbohydrate, polysaccharide, oligosaccharide, sugar, analysis, structure, interaction, diffusion, hydroxy proton.

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Dedication

Till Tereza

The ability to think differently today than yesterday is what separates the wise from the stubborn.

John Steinbeck

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 Morssing Vilén, E., Lundqvist, L.C.E., Jouanneau, D., Helbert, W., Sandstöm, C., (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(12), 3487-3494.

II Morssing Vilén, E., Klinger, M., Sandström, C., (2011). Application of diffusion-edited NMR spectroscopy for selective suppression of water signal in the determination of monomer composition in alginates. *Magnetic Resonance in Chemistry* 49(9), 584-591.

III Ulmer, J., Morssing Vilén, E., Babu Namburi, R., Benjdia, A., Beneteau, J., Malleron, A., Bonnaffé, D., Driguez, P.A., Descroix, K., Lassalle, G., Le Narvor, C., Sandström, C., Spillmann, D., Berteau, O., The first bacterial glycosaminoglycan endosulfatase reveals novel metabolic pathways in the prominent human gut symbiont *Bacteroides thetaiotaomicron*. Manuscript to be submitted to *Journal of Biological Chemistry*.

IV Morssing Vilén, E., Sandström, C., (2013). NMR study on the interaction of trehalose with lactose and its effect on the hydrogen bond interaction in lactose. *Molecules* 18(8), 9735-9754.

Work not included in this thesis:

V Morssing Vilén, E., Kaur, M., Moerschbacher, B., Sandstöm, C., Characterization of the interaction between chitin and chitin binding proteins, BtCBP and BliCBP from *Bacillus thuringiensis* and *Bacillus licheniformis* by SEM, FTIR and ^{13}C cross polarization MAS NMR. Manuscript.

The contribution of Eric Morssing Vilén to the papers included in this thesis was as follows:

I Planning and discussion of project together with co-authors. Majority of planning and execution of experimental work. Majority of the data analysis and the writing.

II Planning and discussion of project together with co-authors. Planning and execution of experimental work. Data analysis and writing of manuscript.

III Discussion of project with Olivier Berteau, Jonathan Ulmer and Corine Sandström. Planning and execution of all NMR-related experimental work. Data analysis and writing of NMR-related results and discussion in the manuscript.

IV Initiation and planning of project together with Corine Sandström. Planning and execution of all experimental work. Data analysis and writing of manuscript.

Abbreviations

BPP-LED	Bipolar gradient Pulse Pair Longitudinal Eddy current Delay
COSY	COrrelation SpectroscopY
CP-MAS	Cross Polarization Magic-Angle Spinning
CS	Chondroitin Sulfate
DOSY	Diffusion Ordered SpectroscopY
DS	Dermatan Sulfate
G	Guluronic acid in alginate
GAG	GlycosAminoGlycan
Gal	Galactose
GalNAc	*N*-acetylgalactosamine
Glc	Glucose
GlcA	Glucuronic Acid
GlcNAc	*N*-acetylglucosamine
HA	Hyaluronic Acid
HMBC	Heteronuclear Multiple Bond Correlation
HMW	High Molecular Weight
HR-MAS	High Resolution Magic-Angle Spinning
HS	Heparin/heparan Sulfate
HSQC	Heteronuclear Single Quantum Coherence
IdoA	Iduronic Acid
KS	Keratan Sulfate
LMW	Low Molecular Weight
M	Mannuronic acid in alginate
M/G-ratio	Mannuronic acid/Guluronic acid - ratio
MM	Molecular Mechanics
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser Effect
NOESY	Nuclear Overhauser Effect SpectroscopY
PFG-NMR	Pulsed Field Gradient NMR

*p*NP-S	Para-Nitro-PhenolSulfate
ROE	Rotating frame nuclear Overhauser Effect
ROESY	Rotating frame nuclear Overhauser Effect SpectroscopY
SD	Standard Deviation
STD NMR	Saturation Transfer Difference NMR
TOCSY	TOtal Correlation SpectroscopY

1 Introduction

The work in this thesis centers on carbohydrates and the use of nuclear magnetic resonance (NMR) spectroscopy to study their structures and properties as well as their interactions with other carbohydrates or biomolecules such as proteins. Carbohydrates of different sizes and classes have been investigated, from common naturally occurring disaccharides through synthesized oligosaccharides up to large polysaccharides, of both plant and animal origin. Most of the work has been done within the framework of the European Union project PolyModE (POLYsaccharide MODifying Enzymes).

Figure 1. The PolyModE project official logotype and project details.

1.1 PolyModE

The overall goal of the PolyModE project was to identify novel polysaccharide modifying enzymes to optimize the potential of hydrocolloids for food and medical applications. The project was concentrated on six polysaccharide classes with high potential for biological and biotechnological applications: alginates, carrageenans, chitosans, glycosaminoglycans (GAGs), pectins and xanthan gums. These six polysaccharides are all of great commercial interest with high current or predicted market value in the multi-billion dollar range. Some of these polysaccharides with the most useful properties are often produced by a few very specific organisms, so that their supply is extremely limited. Their amounts are also continuously reduced due to overutilization and climate changes.

The aim was thus to identify, analyze and then produce enzymes that can be used to increase production of the polysaccharides with the best properties but that are limited in natural supply. Further the identification of novel enzymes that modify polysaccharides in a non-random manner would also produce polysaccharides with new properties and thereby new applications. The main focus was on finding enzymes for modification of substitution patterns and sequence specific depolymerizing enzymes.

A part of the project was dedicated to generic techniques for analysis of the activity and specificity of the new enzymes and to determine the structures and properties of the precursors and of the modified oligo- and polysaccharides. The research in this thesis, within the PolyModE project, has been focused on structural analysis of carrageenans, alginates and GAGs.

Besides the increased availability of raw materials and the generation of novel compounds with new and improved properties the identification of polysaccharide modifying enzymes and the development of methods for their use in production could lead to several advantages compared to current methods of production: reduction of production costs, reduction of energy input and reduction of potentially harmful wastes (PolyModE, 2013).

1.2 Carbohydrates

Carbohydrates together with proteins, nucleic acids and lipids constitute the four major classes of biomolecules. Of these four, carbohydrates are by far the most abundant. Carbohydrates are ubiquitous and serve for example as nutrients, play important roles in cellular recognition, bacterial and viral infections and they are also important as structural material in cell walls and insect and shell fish exoskeletons (Lindhorst, 2007).

Carbohydrates are very diverse and can exist as mono-, oligo- and polysaccharides. Monosaccharides are the smallest carbohydrate building blocks and exist as aldoses and ketoses. A monosaccharide can be found in the two different enantiomeric forms D and L, based on the absolute configuration at the highest numbered chiral center in relation to D-glyceraldehyde. Most common are monosaccharides with five (pentose) or six (hexose) carbon atoms and they are in an equilibrium between an open form and cyclic hemiacetals, formed between the carbonyl group and the hydroxy group on carbon 4 or 5, yielding furanoses and pyranoses respectively. Upon cyclization a new stereogenic center is formed, called the anomeric center. The hydroxy group at the anomeric center can be in either β or α position and because of the equilibrium between the open and closed forms the configurations can interchange. The 6-membered ring of aldohexose monosaccharides has two

distinct forms of the most common conformation, the chair conformation. These two forms are $^4C_1$ and $^1C_4$ which indicate whether carbon-1 and carbon-4 are above or below the reference plane of the chair conformation (Figure 2).

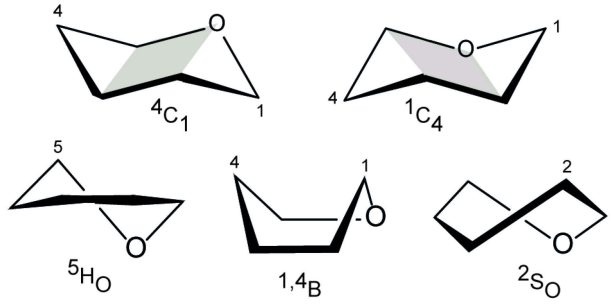

Figure 2. Selected pyranose conformations. Two chair conformations, one half-chair, one boat and one skew-boat conformation.

Different hexoses energetically favor the two forms differently depending on the number of non-bonded interactions and the number of exo-cyclic groups in equatorial versus axial positions (Kamerling *et al.*, 2007). These different preferences for the two forms can also give implications on a macromolecular organizational level of polysaccharides, *i.e.* it can affect helix forming ability and in turn also gelling behavior (Draget *et al.*, 2002; van de Velde & De Ruiter, 2002; van de Velde *et al.*, 2002). In for example alginates the two constituent monosaccharides β-D-mannuronic acid and α-L-guluronic acid have the $^4C_1$ and $^1C_4$ conformations respectively leading to different chain conformations and gelling behavior. Other important conformations of pyranoses are the half-chair (H), boat (B) and skew (S) conformations (Figure 2). Some monosaccharides have more than two low energy conformations that can be observed by NMR in solution. For example iduronic acid have three such conformations, these are $^1C_4$, ^2S_O and $^4C_1$. When internally positioned in oligomeric or polymeric GAGs the $^1C_4$ and ^2S_O conformations are predominant (Ferro *et al.*, 1990).

Disaccharides are dimers of monosaccharides that are formed when the hemiacetal moiety of one monomer reacts with any hydroxy group of another monomer forming an acetal. The bond linking the two sugars is called a glycosidic bond. Disaccharides can be reducing or non-reducing, depending on if they have a free hydroxy group on the anomeric carbon or not. Two of the most well-known disaccharides are sucrose and lactose. Sucrose and lactose are also known to protect and stabilize proteins and other biostructures when

they are for example dried or frozen (Crowe *et al.*, 1998; Crowe *et al.*, 1996). Another disaccharide usually considered superior to sucrose and lactose in this respect is trehalose (Green & Angell, 1989). Many draught resistant, anhydrobiotic, organisms are known to accumulate trehalose to protect their biostructures (Crowe *et al.*, 1998). Trehalose and sucrose are non-reducing disaccharides and lactose is reducing, all with the same molecular formula ($C_{12}H_{22}O_{11}$). They are comprised of α-D-glucopyranosyl-(1↔1)-α-D-glucopyranoside, α-D-glucopyranosyl-(1↔2)-β-D-fructofuranoside and β-D-galactopyranosyl-(1→4)-D-glucopyranose respectively (Figure 3).

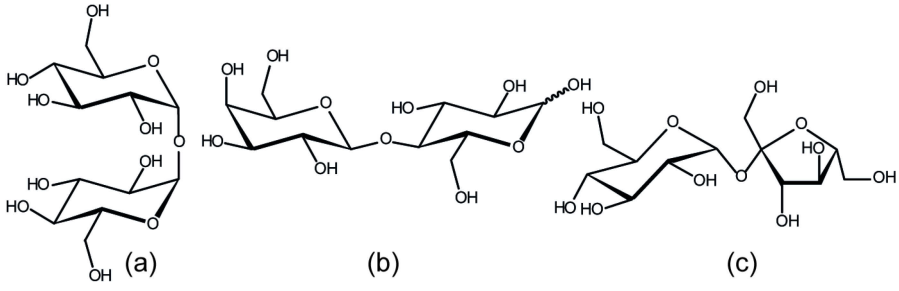

Figure 3. Schematic structures of (a) trehalose, (b) lactose and (c) sucrose.

When two to ten monosaccharides are linked together via glycosidic bonds they are termed oligosaccharides, larger structures are polysaccharides (Kamerling *et al.*, 2007). Around $4x10^{11}$ tons of carbohydrates are biosynthesized in nature each year, the majority of this is produced as polysaccharides. The best known polysaccharides are the structural polysaccharides cellulose and chitin and the energy storage polysaccharides starch and glycogen (Lindhorst, 2007).

The aforementioned diversity of carbohydrates stems from the number of possible combinations of monosaccharide units and linkages between them. The monosaccharides units can differ in number and position of substituents, absolute configuration (D- or L-), anomeric configurations (α or β) and ring forms (pyranosides, furanosides). In addition, the carbohydrates can form both linear and branched oligomers and polymers.

1.2.1 Carrageenans

Carrageenan is the generic name for a family of linear sulfated galactans found as structural polysaccharides in red algae (*Rhodophyceae*). They are composed of (1–3) linked β-D-galactopyranose (G) and (1–4) linked α-D-galactopyranose (D) which forms the basic repeating unit of carrageenans. There are six major classes of carrageenans; mu (μ), kappa (κ), nu (ν), iota (ι), lambda (λ) and

theta (θ). Different species of algae produce different types of carrageenan, it can also depend on season of harvest and the life cycle stage of the algae (Rinaudo, 2008; van de Velde & De Ruiter, 2002; van de Velde *et al.*, 2002). The classification divides the carrageenans into groups depending on the occurrence of sulfate groups and 3,6-anhydro bridges in the main repeating dimeric units (Figure 4).

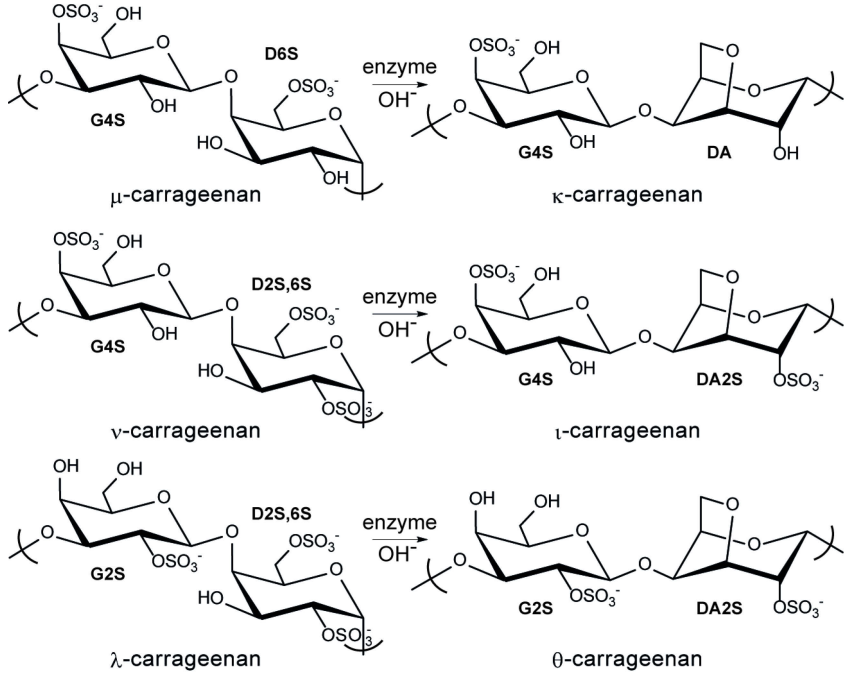

Figure 4. Schematic representation of the different dimeric units in carrageenan. The nomenclature developed by Knutsen *et al*. (1994) can be found for all monomeric units.

μ, ν and λ are the biological precursors of κ, ι, and θ respectively. The different classes represent idealized dimeric structures, the polysaccharides themselves are not totally homogenous (van de Velde *et al.*, 2002). A simple nomenclature to describe the complex structures was developed by Knutsen *et al*. (Knutsen *et al.*, 1994) where G denotes a (1–3) linked β-D-galactose and D a (1–4) linked α-D-galactose. A represents the existence of a 3,6-anhydro bridge and S preceded by a number designates the position of sulfation. Only the (1–4) linked α-D-galactose units can have an anhydrobridge (Figure 4). The dimeric units in the different carrageenans are called carrabiose or neocarrabiose, depending on whether there is a G or a D residue in the reducing end (Figure 5).

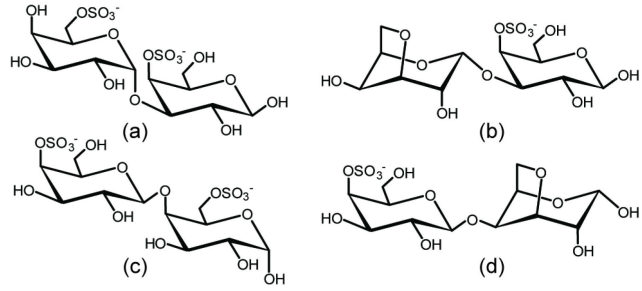

Figure 5. Examples of carrabiose and neocarrabiose dimeric structures. a) μ-β-neocarrabiose, b) κ-β-neocarrabiose, c) μ-α-carrabiose and d) κ-α-carrabiose.

The commercially most important carrageenans are ι, κ and λ, where the two former are gelling agents and the latter is a viscosity enhancer (van de Velde *et al.*, 2002). Carrageenans are used in, for example, frozen desserts, jellies, cottage cheese and sauces. Other areas of use are pharmaceutical products and lubricants for the mining industry. The size of commercial carrageenans is usually in the range of 400–600 kDa, with a lower limit of use in food products of 100 kDa (van de Velde & De Ruiter, 2002).

The carrageenan polysaccharides are usually extracted from the algae either through hot water or hot alkaline extraction. With alkaline extraction there is a simultaneous conversion of μ, ν and λ into the gelling anhydro bridge forms κ, ι and θ (van de Velde *et al.*, 2002). The mechanisms of gelling differ slightly between the different types of carrageenan. It is considered that carrageenan gelling starts with that the polysaccharide chains transform from random coil structures into helices (double or single) that then aggregate into gels. The exact mechanisms for this is however not yet fully understood (Michel *et al.*, 2006), while it is known that the ionic strength and the salts in solution greatly affect the gelling properties of carrageenans. The precursor carrageenans μ, ν and λ have reduced, or no, gelling ability due to the $^4C_1$ conformation of the D-residues which introduce kinks in the polysaccharide chain and hinders helix formation which in turn impedes gelling (van de Velde *et al.*, 2002) (Figure 6).

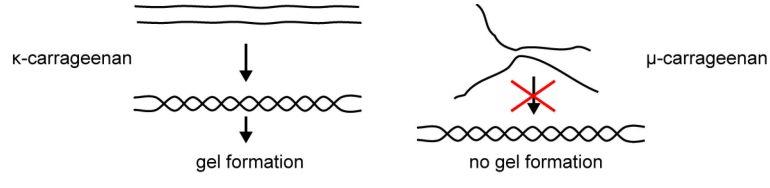

Figure 6. Schematic representation of how the differences in polysaccharide chain conformation affect helix formation and gelling behavior of κ- and μ-carrageenan.

Structural analysis of carrageenans is important because of the different traits of the different types; the right type needs to be used for the right application. Interactions and gelling mechanisms are also important to study, to know how to develop carrageenans with new properties for new applications, and to correlate structures and functions (van de Velde *et al.*, 2002). Enzymes active on carrageenans can greatly aid in the analysis of the polysaccharide. Carrageenases can be used to depolymerize the polysaccharide at defined positions. The resulting oligosaccharides can then be characterized by analytical methods (Jouanneau *et al.*, 2010; Guibet *et al.*, 2006). Sulfatases can be used to control and analyze sulfation and thus gelling behavior (Prechoux *et al.*, 2013). Sulfurylases can introduce the anhydro bridge in D-residues in a specific manner without the depolymerizing side effects of alkali treatment (Genicot-Joncour *et al.*, 2009). Biological studies of carrageenans are important because in the past both positive and negative health effects have been associated with carrageenans (Campo *et al.*, 2009). Antiviral and antitumor activities have been found (Zhou *et al.*, 2004; Carlucci *et al.*, 1999) but studies also show that LMW oligomeric carrageenans can induce inflammation, intestinal ulcerations and neoplasms (Tobacman, 2001).

1.2.2 Alginates

Alginate is a polysaccharide that is found in brown seaweeds (*Phaeophyceae*) and two families of bacteria, *Pseudomonadaceae* and *Azotobacteraceae*. In algae alginate is a cell wall structural component, in *Azotobacter* it is a cell wall component of vegetative capsules and dormant cysts and in *Pseudomonas* it is an exopolysaccharide utilized in biofilms. Alginate is a linear copolymer that consists of (1→4) linked β-D-mannuronic acid (M) and α-L-guluronic acid (G). In the polysaccharide the M and G residues are arranged in three different ways, either as blocks of M or G or alternating M and G (Figure 7) (Haug *et al.*, 1966).

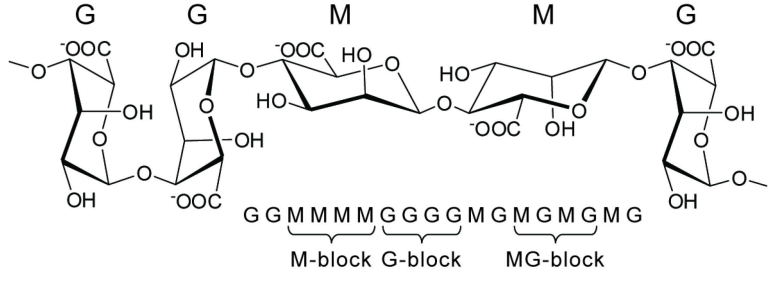

Figure 7. Schematic structure of alginate and a representation of possible block-structures.

The arrangement of M and G residues vary with the seaweed source and harvesting season (Draget *et al.*, 2002). Alginates are easily soluble in water, forming gels or viscous solutions, in the presence of M^{2+} counter ions. They have numerous applications in the food industry to ensure proper texture, density and stability in dairy products, baked goods and restructured food stuffs (Draget *et al.*, 2006). Alginates are biocompatible making them suitable for use in biomedical and pharmaceutical areas such as absorbent in wound dressings and molds in dentistry (Rinaudo, 2008). Different areas of use require different properties of the alginate and its gelling. The physical properties of alginates are correlated to the monomeric composition or M/G-ratio, the M and G distribution and the molecular weight (Stokke *et al.*, 1991; Haug *et al.*, 1967b). Alginates of high G-block content gives strong gels in presence of Ca^{2+} ions where the alginates form junction zones by binding to the Ca^{2+} ions, the egg box model (Figure 8) (Grant *et al.*, 1973). Alginates with high M-block or alternating structure content form more disordered conformations and hence more flexible gels. Commercial alginates vary greatly in size, between 32 and 400 kDa, depending on the application, generally higher molecular weight alginates give stronger gels (Rinaudo, 2008). Alginate with high G content is an excellent replacement for gelatin in many restructured foodstuffs, because of its plant rather than animal origin.

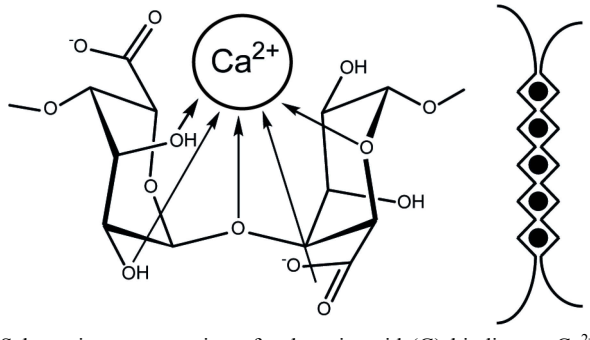

Figure 8. Left: Schematic representation of guluronic acid (G) binding to Ca^{2+} in the egg-box model. Right: Overview of the egg-box model. Black circles represent Ca^{2+} ions and solid lines the alginate polymer.

Alginates with M/G-ratios of 0.5–0.7 are in high demand from industry but can be difficult to obtain from natural sources since many seaweed species only produce alginates with M/G-ratios around 1.2–1.6. Manipulating the M/G-ratio of alginates can be done chemically but requires high temperatures or catalysts that yield depolymerized or catalyst contaminated alginates. Enzymatic alteration of M/G-ratios is also possible using C5-epimerases that catalyze the conversion of M into G in the alginate polymer (Draget *et al.*, 2006; Valla *et*

al., 2001; Ertesvåg *et al.*, 1999). Non-chemical and sequence specific depolymerization of alginates can be achieved with lyases (Thomas *et al.*, 2013; Aarstad *et al.*, 2012; Lundqvist *et al.*, 2012; Ostgaard *et al.*, 1993; Romeo & Preston, 1986). C5-epimerases used in combination with chemical hydrolysis or sequence specific alginate lyases can generate the substrate specificity of the epimerase and also, in the latter case, sequence specific oligomers. Since alginates from different algal sources have different M/G-ratios and show very different properties it is important to be able to determine this ratio accurately, to ensure proper characterization of the alginate. This is usually done by NMR (Grasdalen, 1983; Grasdalen *et al.*, 1981; Grasdalen *et al.*, 1979a; Grasdalen *et al.*, 1977; Penman & Sanderso.Gr, 1972) but IR, near-IR and Raman spectroscopy are also used (Salomonsen *et al.*, 2008; Sakugawa *et al.*, 2004; Mackie, 1971).

1.2.3 Glycosaminoglycans

Glycosaminoglycans (GAGs) are a large group of heterogeneous, linear, negatively charged polysaccharides that can be divided into five different subfamilies: chondroitin, heparin/heparan sulfates, dermatan, keratan and hyaluronic acid. Of those five groups the first four are sulfated proteoglycans whereas hyaluronic acid is non-sulfated and not attached to any protein. All the GAGs have different disaccharide repeating units which both within and between the groups can differ in sulfation pattern, meaning that GAGs potentially are more information dense than DNA or proteins (Sasisekharan *et al.*, 2006). The heparin and heparan sulfate (HS) repeating disaccharide unit is (→4)α-L-IdoA/β-D-GlcA(1→4)α-D-GlcN(1→) while chondroitin sulfate (CS) consists of (→4)β-D-GlcA(1→3)β-D-GalNAc(1→). The basic building block of dermatan sulfate (DS), which is similar to chondroitin, is made of (→4)α-L-IdoA(1→3)β-D-GalNAc(1→). Keratan sulfate (KS) lacks any hexuronic acid and consists of (→3)β-D-Gal(1→4)β-D-GlcNAc(1→). The fully unsulfated GAG hyaluronic acid (HA) has the repeating unit; (→4)β-D-GlcA(1→3)β-D-GlcNAc(1→). For a detailed view, and possible sulfation patterns, of the different GAGs see figure 9.

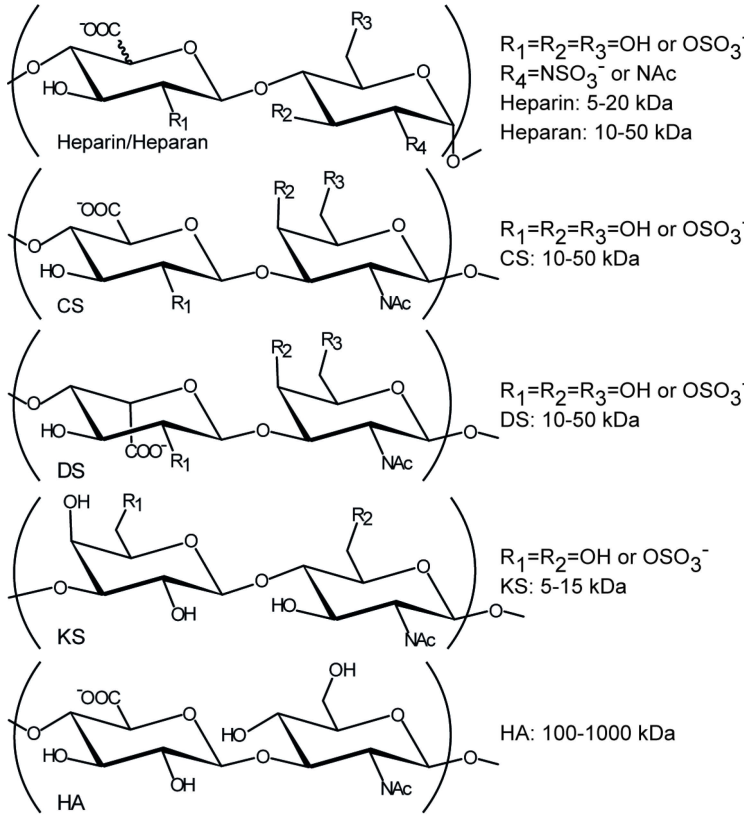

Figure 9. Schematic representation of GAG structures. Sulfate groups can be present at all or some of the specified positions.

GAGs are present in all animals (metazoan) and in some bacteria (DeAngelis, 2012). In vertebrates they possess structural (connective tissue such as skin, cartilage, epithelia etc.) and metabolic functions (regulation of cell growth, anti-thrombotic effects, receptor and co-receptor functions). The specific functions vary between the different classes of GAGs (DeAngelis, 2012; Wight, 1999). GAGs are essential and malfunctions in their biosynthesis or regulation can result in a number of diseases such as atherosclerosis, cancer and Alzheimer's disease (Wight, 1999). HA is extensively used in the cosmetics and medical industry, CS is emerging as a valuable nutraceutic and LMW heparins are used for thrombosis treatment.

The study of GAGs is a challenge. Their inherent heterogeneity in both chain length and sulfation, partly a result of their non-template driven biosynthesis, makes it difficult to both isolate and characterize different structures. Structural analysis of GAGs have progressed a lot in recent years due to great efforts in the development of enzymatic tools for specific

depolymerization and modification of GAGs as well as in the development of analytical tools and methods (Sasisekharan *et al.*, 2006).

Important GAG modifying enzymes that can be used for structural analysis or chemo-enzymatic synthesis of GAGs are sulfatases, sulfotransferases and glycosidases. The biological properties of GAGs, such as heparin, are correlated to their structure and sulfation pattern. Identification of new enzymes with the ability to add or remove sulfate groups at specific positions in a specific residue in a GAG chain will allow in conjunction with glycosidases to produce GAGs of defined sizes and with a defined substitution pattern. Enzymatically tailored GAGs are of great value for structure-function studies or for therapeutic purposes since synthesis of GAGs is laborious and difficult and extraction of GAGs from animal sources generally produce small amounts of heterogenic GAG mixtures (DeAngelis *et al.*, 2013). The ability to tailor or structurally characterize GAGs *in vitro* is expected to grow, with the help of specific sulfatases, increasing our ability to study structure-function relationships in detail. It will also help in the production of more specific, more functional and potentially novel GAG structures and therapeutics such as heparin-drugs (DeAngelis *et al.*, 2013; Pempe *et al.*, 2012).

2 NMR Spectroscopy

NMR is today an indispensable analytical tool for many chemists, physicists and biochemists. It all started in the 1940s when the groups of Bloch and Purcell observed nuclear magnetic resonance signals for the first time, in water and paraffin respectively (Bloch *et al.*, 1946; Purcell *et al.*, 1946). The first NMR spectrum that could characterize an organic molecule, ethanol, was obtained in 1951 (Arnold *et al.*, 1951). Since then countless structures, of small, large, simple and complicated molecules have been analyzed and determined, biomolecular interactions have been studied and mapped and NMR spectroscopy is still developing.

The basis of NMR spectroscopy arises from the fact that atomic nuclei, for example ^{1}H, with an angular momentum (P) generate a small magnetic field and thus possess a magnetic moment (μ). The angular momentum can be quantized and given an angular momentum quantum number, I, frequently called spin quantum number or spin. The spin and the magnetic moment are related through the gyromagnetic ratio (γ). The gyromagnetic ratio is a constant for each isotope of each element.

Nuclei for which the spin quantum number is non-zero are NMR active and can in principle be studied by NMR spectroscopy. Nuclei with an odd mass or atomic number have non-zero spin quantum numbers. For a I = ½ nucleus, like ^{1}H, placed in a strong static magnetic field the magnetic moment will align parallel (low energy) or anti-parallel (high energy) to the applied magnetic field, yielding two energy levels. If the nuclei are then subjected to radiofrequency radiation of the proper frequency they will absorb the energy and transfer spins from the lower to the higher energy level. When the excess energy is dissipated a signal can be observed. The frequency of absorption is characteristic of the type of nuclei (^{1}H or ^{13}C etc.) and also depend on the applied static magnetic field and the chemical environment of the nuclei. The difference between resonance frequencies of nuclei in a molecule are very

small and are expressed as the chemical shift (δ), in ppm, compared to a reference compound. Homogeneity of the static magnetic field is important. Small differences in resonance frequencies can be obscured by linebroadening caused by magnetic field inhomogeneity since the resonance frequency of a nucleus is also dependent on its spatial location in an inhomogeneous magnetic field. (Keeler, 2011; Lambert & Mazzola, 2004; Friebolin, 1991). The dependence of the resonance frequency on spatial location in an inhomogeneous magnetic field can be exploited in the measurement of self-diffusion coefficients by NMR (Stejskal & Tanner, 1965).

2.1 NMR Spectroscopy and Carbohydrate Analysis

The study of carbohydrates by NMR spectroscopy can involve structure determination, conformational analysis and determination of interactions between carbohydrates or between carbohydrates and other biomolecules (Widmalm, 2013).

2.1.1 General Techniques and Experiments

Carbohydrates are a group of compounds with vast structural and chemical diversity but with a limited chemical shift dispersion in NMR spectra which makes their study by NMR challenging and intriguing.

The assignments of ^{1}H and ^{13}C resonances to their corresponding protons and carbon atoms are usually achieved using a combination of 1D and 2D NMR experiments such as homonuclear ^{1}H-^{1}H COSY, ^{1}H-^{1}H TOCSY, and ^{1}H-^{1}H NOESY or ^{1}H-^{1}H ROESY experiments and heteronuclear ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC and ^{1}H-^{13}C HSQC-TOCSY.

^{1}H-1D experiments are usually the starting point of the analysis. These experiments give plenty of information despite that most resonances are found in the crowded region between ~3.4 and ~4.0 ppm. Well resolved signals are usually the anomeric protons (4.4–5.5 ppm) and methyl or acetyl protons (~1.2 and ~2 ppm respectively).

The anomeric signals can be used to estimate the number of different monosaccharides present in the sample. Other well resolved signals can come from the presence of for example sulfurylation or phosphorylation. The magnitude of vicinal coupling constants ($^{3}J_{H,H}$) can provide information about the dihedral angles between ring protons (Karplus, 1963) and the peak intensities about the relative number of protons making up each signal.

The next step frequently consists of making use of homonuclear correlation experiments such as COSY and TOCSY. These experiments exploit the scalar couplings between protons in a molecule; through bond correlations of protons

are detected. A COSY spectrum typically shows correlations between geminal or vicinal protons although four-bonded correlations may also be seen in some cases. TOCSY type experiments show correlations between all protons within a given spin system. TOCSY make it possible to simultaneously correlate the entire spin system in a sugar residue, depending on the mixing time of the experiment and the size of the coupling constants between the different protons. COSY and TOCSY experiments normally yield plenty of information on the type of monosaccharides present in a sample. Using well resolved anomeric, or other, signals as starting points it is often possible to "walk" through the bonds of a sugar residue.

In addition to homonuclear correlations, heteronuclear correlations can be used for structure determination. Two commonly used experiments are HSQC and HMBC. HSQC experiments correlate ^{1}H atoms with the ^{13}C atoms they are directly bonded to. The larger chemical shift dispersion for carbon signals is an advantage in structural assignments. HSQC can give a clearer picture of the number of different monosaccharides in the sample since the ^{13}C anomeric signals appear in a characteristic region between ~90 to 110 ppm. Multiplicity edited-HSQC experiments can be used to differentiate CH and CH_3 carbons from CH_2 carbons. The HSQC-TOCSY experiment is a 2D TOCSY experiment resolved into the carbon dimension. In HSQC-TOCSY crosspeaks are seen between all J-coupled protons in a spin system and each carbon in that spin system. The extra dispersion in the carbon dimension can be very advantageous when overlap in the proton dimension prevents analysis. HMBC experiments are used to identify multiple bond correlations, for example to establish the position of quaternary carbons in a structure or to find correlations across glycosidic linkages to determine the linkage between adjacent sugars.

Another way to obtain linkage information is to detect through-space correlations. Magnetization can be transferred through scalar couplings but also through dipolar interactions (the nuclear Overhauser effect, NOE). The NOE arise from dipole-dipole relaxation between spins through space and depends on the internuclear distance and molecular motion. The NOE effect can be observed for protons up to approximately 5 Å apart. (Wagner & Wuthrich, 1979; Gordon & Wuethrich, 1978). Whether to use NOESY or ROESY experiments depends on the size of the molecule, on the temperature at which the experiment is performed, on the viscosity of the solvent and of the strength of the magnetic field. With an increase in molecular size and departure from extreme narrowing limit behavior NOE effects become smaller and finally negative after passing through zero (Figure 10). To avoid zero or very small NOE enhancements the ROESY experiment can be used for small and

medium sized molecules since the ROE is always positive and increase with molecular size (Bax & Davis, 1985; Bothner-By *et al.*, 1984).

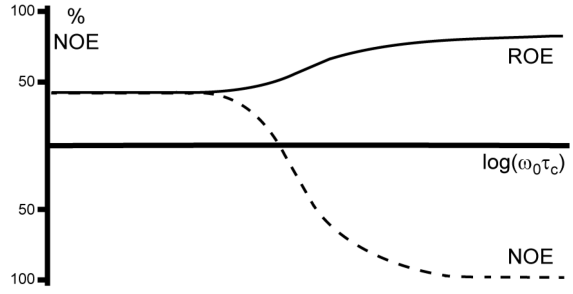

Figure 10. Schematic illustration of the dependence of ROE and transient NOE for a homonuclear isolated two-spin system, as a function of the rate of molecular tumbling.

In this thesis, the experiments mentioned above have been used to obtain ^{1}H and ^{13}C assignments. The following chapters describe other NMR methods and experiments that have also been used in the thesis.

2.2 Hydroxy Protons and NMR Spectroscopy

Carbohydrates are structurally very diverse but the hydroxy group is common to all. These hydroxy groups are important since they are involved in interactions with other biomolecules and with water. Study of hydroxy protons can provide additional NOE constraints for conformational analysis as well as information on hydration and hydrogen bonding interactions (Hakkarainen *et al.*, 2007; Rohfritsch *et al.*, 2007; Hakkarainen *et al.*, 2005; Bekiroglu *et al.*, 2004a; Bekiroglu *et al.*, 2004b; Bekiroglu *et al.*, 2003; Bekiroglu *et al.*, 2000; Sandstrom *et al.*, 1999; Sandstrom *et al.*, 1998b; Sandstrom *et al.*, 1998a; Sheng & Vanhalbeek, 1995; Adams & Lerner, 1994; Bundle *et al.*, 1994; Poppe & Vanhalbeek, 1994; Adams & Lerner, 1992; Poppe *et al.*, 1992; Poppe & Vanhalbeek, 1992b; Poppe & Vanhalbeek, 1992a; Vanhalbeek & Poppe, 1992; Poppe & Vanhalbeek, 1991). The first observation of hydroxy protons of carbohydrates by NMR was reported in 1976 by Harvey and Symons (Harvey *et al.*, 1976).

In this thesis NMR of hydroxy protons was used to study the structure of κ and κ/μ-hybrid carrageenan oligosaccharides as well as the effect of trehalose on the hydration and hydrogen bonding in lactose, in papers I and IV respectively.

2.2.1 Sample Preparation

Carbohydrates studied by NMR spectroscopy in aqueous solution are usually dissolved in deuterated water (D_2O). When studying the exchangeable NH and OH protons, D_2O cannot be used as a solvent because the protons in the sample will exchange with the deuterons from the solvent and become invisible in the NMR spectra (Figure 11).

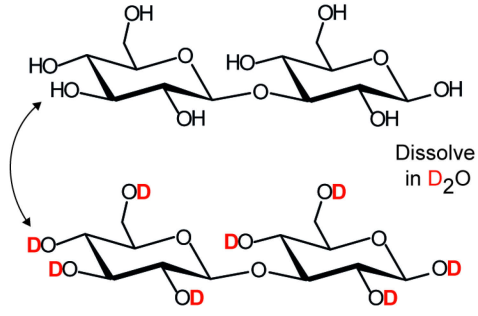

Figure 11. The exchange of hydroxy protons for deuterons when a sugar is dissolved in D_2O.

Instead H_2O, or solvents without exchangeable deuterons, has to be used to study hydroxy protons. The use of H_2O as the solvent is usually not sufficient to make the hydroxy protons visible and their rate of exchange with water has to be lowered. Normally this is achieved by adjusting the pH to 5.5–7 by addition of small amounts of HCl or NaOH. To minimize the release of impurities, like borate ions, from glassware the NMR sample tubes are soaked in 50 mM phosphate buffer (pH 7) for at least one hour (Adams & Lerner, 1992), and subsequently rinsed with deionized water. Further reduction of the rate of exchange is achieved by lowering the sample temperature to below 0 °C. Sub-zero temperatures of aqueous samples are possible by using highly concentrated solutions or super-cooling (Batta & Kover, 1999; Poppe & Vanhalbeek, 1994). However, the most common strategy to avoid freezing of the sample is to add 10–15% of an organic solvent such as acetone or methanol. This mixture permits the temperature to be lowered to about –15 °C without the sample freezing, the exact temperature depending on the concentration of the sugar. The NMR spectra before and after careful sample preparation are shown in Figure 12.

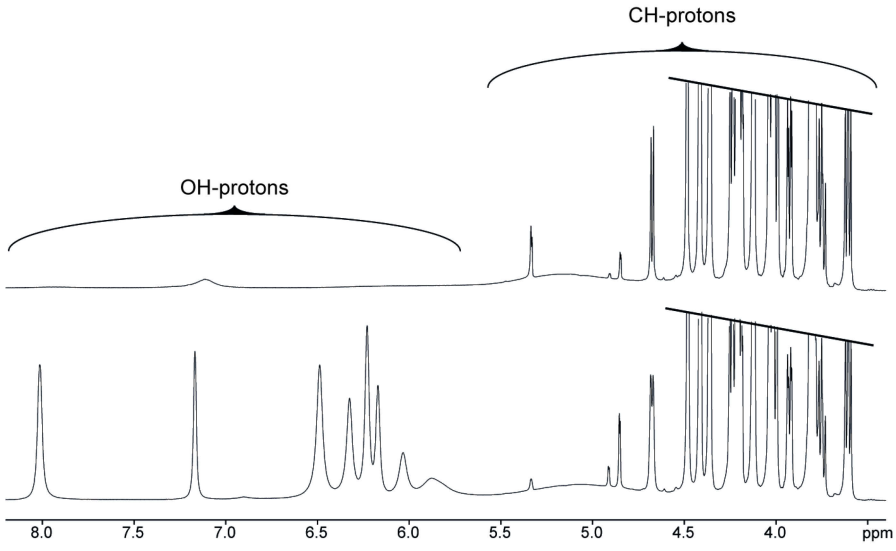

Figure 12. Spectra of κ-neocarrabiose before (top) and after cleaning of NMR tube and pH adjustement (bottom).

The effects of small amounts of the relatively weak hydrogen bond donor $(CD_3)_2CO$ (Gomide Freitas *et al.*, 1999) on hydroxy proton chemical shifts and solvation have been assessed. Another study showed that chemical shifts of hydroxy protons were relatively insensitive to different sample conditions such as pH or the concentration of acetone in water (Adams & Lerner, 1994). In dicarboxylic acids it has been shown that even in 90/10% $(CD_3)_2CO/H_2O$ solutions the amount of water was sufficient to allow for full solvation of the intramolecularly hydrogen bonded species (Lin & Frey, 2000). Also, in β-cyclodextrin hydroxy proton chemical shifts and coupling constants were the same in both 95/5% H_2O/D_2O and 85/15% $H_2O/(CD_3)_2CO$ (Bekiroglu *et al.*, 2003).

2.2.2 Suppression of the Water Signal

The huge water signal can be efficiently suppressed by pulsed-field gradient (PFG) experiments such as WET (Smallcombe *et al.*, 1995; Ogg *et al.*, 1994), WATERGATE (Liu *et al.*, 1998; Sklenar *et al.*, 1993; Piotto *et al.*, 1992) and excitation sculpting (Hwang & Shaka, 1995). Water presaturation cannot be used because hydroxy protons exchanging with water will also be saturated. WATERGATE, or variants thereof, and excitation sculpting are incorporated into many *n*D experiments.

2.2.3 Hydroxy Proton NMR Parameters

There are a number of hydroxy proton NMR parameters that can give information on the structure, conformation, hydrogen bonding interactions and hydration of carbohydrates.

Chemical shifts (δ) and chemical shift differences (Δδ)

The chemical shifts of hydroxy protons in aqueous solution are usually found between 5.5 and 8.5 ppm, a well isolated region of the NMR spectra downfield from the aliphatic protons. By comparing the chemical shifts of the hydroxy protons in an oligosaccharide with those from its corresponding monosaccharides the chemical shift difference is obtained, $\Delta\delta$ ($\Delta\delta = \delta_{oligo} - \delta_{mono}$). The chemical shift difference can be used as a conformational probe to study hydrogen bond interaction, hydration and spatial proximity to other hydroxy protons, ring oxygen or bulky substituents (Bekiroglu *et al.*, 2004b; Ivarsson *et al.*, 2000; Sandstrom *et al.*, 1998b). Hence a positive $\Delta\delta$ indicates that the hydroxy proton in an oligosaccharide is downfield shifted compared to the shift in the monosaccharide and this reflects spatial proximity to another hydroxy proton. A negative $\Delta\delta$ indicates spatial proximity to non-protonated O5 oxygen or to a bulky substituent and is attributed to a reduced hydration of the hydroxy proton in the oligosaccharide compared to the hydration in the constituent monosaccharide. Hydroxy protons involved in hydrogen bonding should be deshielded (Poppe & Vanhalbeek, 1994) but in strongly hydrated systems such as carbohydrates it appears that the chemical shift of a hydroxy proton is a balance between downfield shifts due to hydrogen bonding and upfield shifts due to reduced hydration (Bekiroglu *et al.*, 2004b).

Vicinal $J_{CH,OH}$ coupling constants

Coupling constants between hydroxy protons and aliphatic ring protons can be used to determine H-C-O-H dihedral angles. According to the Karplus equation derived for hydroxy protons vicinal coupling in the order of 5.5 ± 0.5 Hz indicates conformational averaging with free rotation for the hydroxyl group around the C–O bond (Fraser *et al.*, 1969). A hydroxy proton with a $^3J_{CH,OH}$ that significantly deviates from 5.5 ± 0.5 Hz would indicate restricted rotation around the H-C-O-H bond which in turn can be an indication of involvement in a hydrogen bonding interaction.

Temperature coefficients (dδ/dT)

The chemical shifts of hydroxy protons that are only hydrogen bonded to the solvent have marked temperature dependence due to changes in mobility of the solvent molecules. The chemical shifts of other hydrogen-bonded hydroxy

protons are less affected by temperature due to the decreased interaction with solvent. The dδ/dT is obtained by measuring the chemical shifts of the hydroxy protons at several different temperatures, usually in the range –15 to +15 °C.

Hydroxy protons that are strongly solvated generally have large absolute values of temperature coefficients, above 10 ppb/°C, while hydroxy protons involved in strong intramolecular hydrogen bonds are expected to have absolute values of dδ/dT below 3 ppb/°C (Kroon *et al.*, 1994; Poppe *et al.*, 1992; Poppe *et al.*, 1990a). For trisaccharides in aqueous solution $|d\delta/dT|$ of 4 ppb/°C and lower have been reported (Sandstrom *et al.*, 1998b; Poppe *et al.*, 1992) and for sugars in DMSO solution 3 ppb/°C has been taken as an indication of involvement in a strong hydrogen bond interaction (Poppe *et al.*, 1990b) Temperature coefficients above 5 ppb/°C can be indicative of weak hydrogen bonding interactions given that other NMR data also corroborate the interaction.

Rotating frame nuclear Overhauser effect (ROE)

When studying hydroxy protons ROESY experiments are used to distinguish between crosspeaks due to dipolar relaxation and chemical exchange. Crosspeaks due to chemical exchange will have the same sign as the diagonal peaks while those due to dipolar relaxation will have the opposite sign (Davis & Bax, 1985). Chemical exchange between hydroxy protons can be an indication of spatial proximity of hydroxy groups and have been used to identify weak hydrogen bonding interactions in sucrose (Sheng & Vanhalbeek, 1995).

Other NMR approaches that can be used to investigate hydrogen bonding and hydration in carbohydrates are determination of rate of exchange for hydroxy protons with water (Kumar *et al.*, 1981; Jeener *et al.*, 1979), line width analysis of hydroxy proton signals (Langeslay *et al.*, 2012), deuterium-induced differential isotope shift, analysis of $J_{C,OH}$ coupling constants (Zhang *et al.*, 2009) and determination of ^{1}H, ^{2}H or ^{17}O relaxation rates of water and sugar protons (Aroulmoji *et al.*, 2012). Since these approaches were not used in the thesis, they will not be further discussed.

2.3 Diffusion and NMR Spectroscopy

It has been possible to study diffusion, or random translational motion of molecules in solution by NMR since the early days of the technique (Carr & Purcell, 1954; Hahn, 1950). The possibility to study translational motion is due to the fact that nuclear spins can be spatially encoded through the use of magnetic field gradients. The first measurements used continuous gradients but

as shown first by Stejskal and Tanner (1965) pulsed field gradient NMR (PFG-NMR) is advantageous in many ways (Figure 13). Some of these advantages are: no line broadening because the gradient is off during acquisition, smaller diffusion coefficients can be measured since the use of larger gradient is possible, the time period for diffusion measurement is well defined which is important for accuracy and measurements of restricted diffusion (Price, 1997).

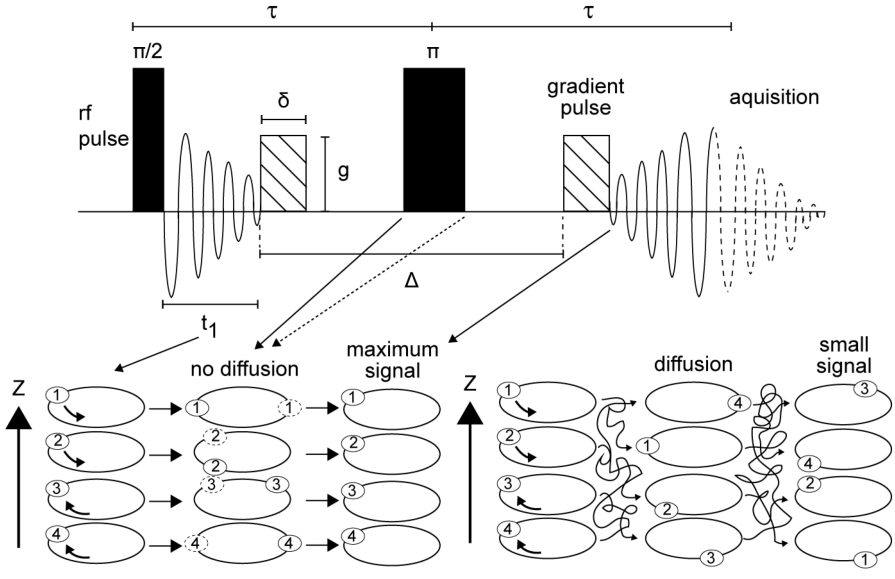

Figure 13. Simplified description of the pulsed field gradient spin echo sequence and the spatial encoding of spins. The situation for non-diffusing or slow-diffusing molecules is described by the left hand part of the figure. Faster diffusion is described by the right hand part of the figure. Figure adapted from Price (1997).

Basically, the translational movement of a molecule between two gradient pulses is detected. The gradients are applied during the defocusing and refocusing parts of a pulse sequence. Slow-moving molecules will experience almost the same field strength at both times and become refocused and signal intensity will therefore decay slowly. Fast-moving molecules will on the other hand experience many different magnetic field strengths during the course of the experiment and thus display incomplete refocusing and rapid signal decay (Figure 13). The attenuation of the signal in the spin echo can be used to calculate the diffusion coefficient of a molecule when the gradient pulse duration, amplitude and the delay between the pulses are known. The differences in translational motion of different molecules can be used for spectral editing (Liu *et al.*, 1996) resolving or discriminating between signals from molecules of different sizes at different experimental conditions. Spectral

editing can also include solvent suppression where the sizes of the solvent and solute molecules differ enough in size (Esturau & Espinosa, 2006; Giernoth & Bankmann, 2005; van Zijl & Moonen, 1990).

In the early 1990s the idea of using translational motion as an extra dimension in a NMR spectrum was realized with the advent of diffusion ordered 2D NMR spectroscopy (DOSY) (Morris & Johnson, 1992). DOSY experiments separate molecules according to their diffusion coefficients in the second diffusion dimension (Figure 14). The applications of DOSY are numerous and include mixture analysis (biofluids and compound libraries etc.), hydrophobicity separation, compartmentalization analysis, molecular association analysis and ligand screening (Johnson, 1999).

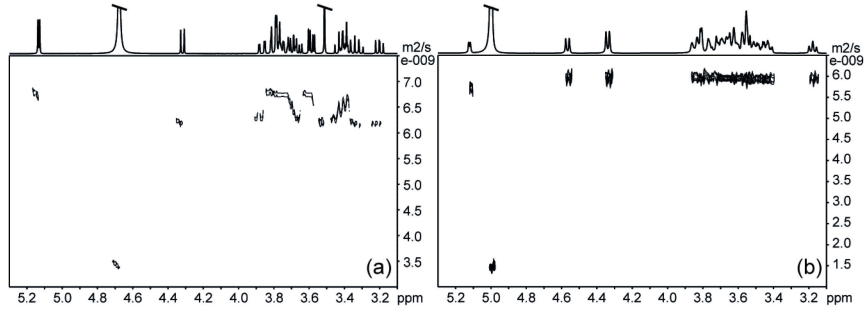

Figure 14. Two examples of DOSY spectra. a) Trehalose and methyl-β-D-glucose in D_2O at 30 °C. b) Lactose in D_2O at 0 °C.

PFG-NMR was used in papers II and III for solvent suppression and spectral editing purposes. In paper IV, PFG-NMR was used to study the diffusion of water and disaccharides in binary and ternary solutions.

2.4 Saturation Transfer Difference NMR Spectroscopy

Saturation transfer NMR has for a long time been used to characterize the binding in ligand-receptor complexes. Carbohydrate-protein binding was analyzed already in the late 1970s (Akasaka, 1979). However the concept of saturation transfer difference NMR (STD NMR) as a means for screening of compound mixtures and for epitope mapping is relatively new and was realized in the late 1990s (Mayer & Meyer, 2001; Maaheimo *et al.*, 2000; Klein *et al.*, 1999; Mayer & Meyer, 1999). The development was partly driven by a need and a will to non-invasively and relatively fast analyze ligand binding to proteins from mixtures of ligands.

STD NMR is based on the selective saturation of the macromolecule NMR signals. The saturation spreads through all the macromolecule NMR signals via spin diffusion, which is particularly effective in macromolecules, like proteins, with tightly dipole-dipole coupled protons. Then, if one ligand out of a mixture is bound to the macromolecule under the conditions of fast exchange the saturation is transferred to that ligand through intermolecular saturation transfer. When the ligand is released from the macromolecule, the transferred saturation can be detected in solution. The resulting spectrum is then subtracted from a spectrum where the macromolecule signals have not been saturated and consequently not the ligand either. This gives a difference spectrum only showing the signals from the ligand that was bound to the macromolecule. The protons in closest contact to the macromolecule will receive most saturation and will yield larger signal in the difference spectrum which makes it possible to map the binding epitope of a ligand at the atomic level (Mayer & Meyer, 2001). The principle is schematically described in figure 15.

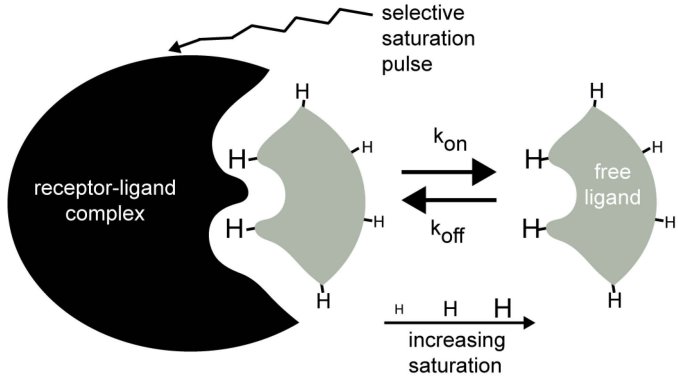

Figure 15. Schematic description of the STD NMR experiment and its use in epitope mapping. Figure adapted from Mayer & Meyer (2001).

Very low amounts of the macromolecule (low μM range) are required since it is the ligand, usually in 50 to 100-fold excess, which is detected. Knowledge of the macromolecule structure or isotope labeling of it is not needed and there is no upper size limit for the macromolecule. Ligands with dissociation constants in the high mM to nM range (10^{-3}–10^{-8} M) can be studied and binding constants can be obtained. The STD scheme can be incorporated into NMR experiments, such as COSY, TOCSY, NOESY and HSQC. Drawbacks include difficulty to differentiate between strong and non-binders since they both give no signals in an STD spectrum. However this problem can be solved by performing a competition study with a known weak binder. Also extra care has to be taken to ensure full saturation when the macromolecule is small (Claasen

et al., 2005; Meyer & Peters, 2003; Mayer & James, 2002; Mayer & Meyer, 2001; Klein *et al.*, 1999; Mayer & Meyer, 1999). STD NMR was used in paper III to study the binding between a GAG sulfatase enzyme and a synthetic substrate.

2.5 Specific Aims

In the first project the structural implications of having μ-residues in κ-carrageenan oligosaccharides were investigated.

The aim of the second project was to find a reliable method for measuring the M/G-ratio on intact alginate samples in solution. Such a method should require limited sample preparation and be applicable to the screening of large sample sets.

The third project was aimed at identifying novel bacterial sulfatases and determining the specificity of these enzymes against different types of GAG oligo- and polysaccharides.

In the fourth project, the effects of trehalose and sucrose on the hydration properties and hydrogen bonding of lactose were investigated.

3 Results and Discussion

This chapter of the thesis is a summary of the results found in papers I–IV. Some results not presented in the papers are also introduced and discussed.

3.1 Paper I: 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

The structure and properties of carrageenans greatly depend on the type of carrageenan. κ-Carrageenan is a gelling polysaccharide whereas its biological precursor, μ, is non-gelling. κ-Carrageenan exists as a mixture of κ and μ-carrageenan since it is biosynthesized from μ-carrageenan. The mixture of κ and μ-carrageenan hampers helix-forming ability and in turn the gel-forming capacity. From a perspective where consistency in gelling is a key factor, understanding the structure-function relationships is important. The *in vivo* transformation of μ into κ-carrageenan is performed by sulfurylase enzymes and *in vitro* by hot alkali treatment. The incorporation of the 3,6-anhydrobridge into the D-residues stabilizes the $^{1}C_{4}$ chair conformation and thus the gelling abilities of the carrageenans. The lower jelling ability of μ-carrageenan stems from the $^{4}C_{1}$ conformation of the D-residues that causes kinks in the polysaccharide chain that impede helix formation and subsequent gelling.

The structures of κ and κ/μ oligosaccharides produced by enzymatic digestions of carrageenans from cultivated *Kappaphycus alvarezii* were recently solved by NMR spectroscopy (Jouanneau *et al.*, 2010).

In this study, unusual downfield shifts of the anomeric protons of the D-galactose-6-sulfate sugar (H1_D6S[1]) were observed when one μ-neocarrabiose unit was positioned between two others. We hypothesize that such a downfield shift could originate from differences in hydrogen bonding interactions for the different oligosaccharides. To evaluate this hypothesis a study of the hydroxy protons by NMR spectroscopy was performed.
The κ and κ/μ-carrageenan oligosaccharides and their constituent mono saccharides shown in figure 16 were investigated.

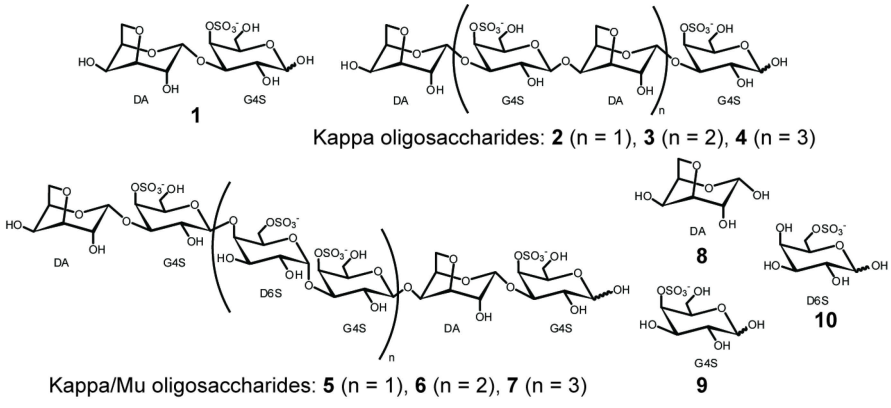

Figure 16. Structures of the mono- and oligosaccharides **1**–**10**.

The chemical shifts (δ), chemical shift differences (Δδ), temperature coefficients (dδ/dT), NOEs and chemical exchange of hydroxy protons were measured to gain insight into hydrogen bonding and hydration. Due to broad hydroxy proton signals and spectral overlap the coupling constants, $^{3}J_{CH,OH}$, could be measured only for compounds **1**, **2** and **8**. The spectral overlap and broad signals in the larger oligosaccharides precluded the measurement of hydroxy proton exchange rates with water. The assignments of proton resonances were made using ^{1}H-1D, DQF-COSY and TOCSY 2D spectra as well as previously recorded data (Jouanneau *et al.*, 2010; Knutsen & Grasdalen, 1992). The hydroxy proton resonances being in a well isolated region of the NMR spectra were used for the assignment of H5 and H6 whose assignment could not be obtained previously (Figure 17).

1. H1 describes the proton attached to carbon 1 of that sugar, D describes the type of sugar, 6 and S after D indicates a sulfation at position 6 of that residue. A number in parenthesis at the end specifies the number of that monosaccharide in the oligosaccharide chain, counting from the reducing end.

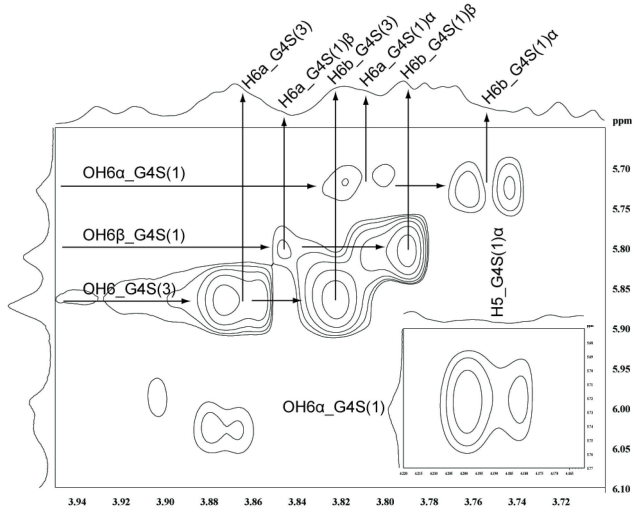

Figure 17. Part of a TOCSY spectrum of κκκκ **4** showing the possibility to assign aliphatic proton shifts of sugars through correlation to hydroxy protons.

For the κ-oligosaccharides there was no experimental evidence of the existence of strong hydrogen bonding interactions. The absolute values of Δδ of the hydroxy protons in the oligosaccharides were small (< 0.2 ppm) indicating hydration similar to that in the constituent monosaccharides. Most hydroxy protons had large negative temperature coefficients (Figure 18). No interresidual NOEs between hydroxy protons or between hydroxy protons and aliphatic protons were found. Only the negative Δδ and slightly lower dδ/dT of all OH2_G4S compared to those of OH2_DA might reflect reduced hydration due to spatial proximity to the ring oxygen of DA. A hydrogen bond between OH2 of G4S and O5 of DA has indeed been found in the crystal structure of neocarrabiose and by simulations using MM force fields (Bosco *et al.*, 2005; Stortz & Cerezo, 2003; Ueda *et al.*, 2001; Stortz & Cerezo, 2000; Lamba *et al.*, 1990).

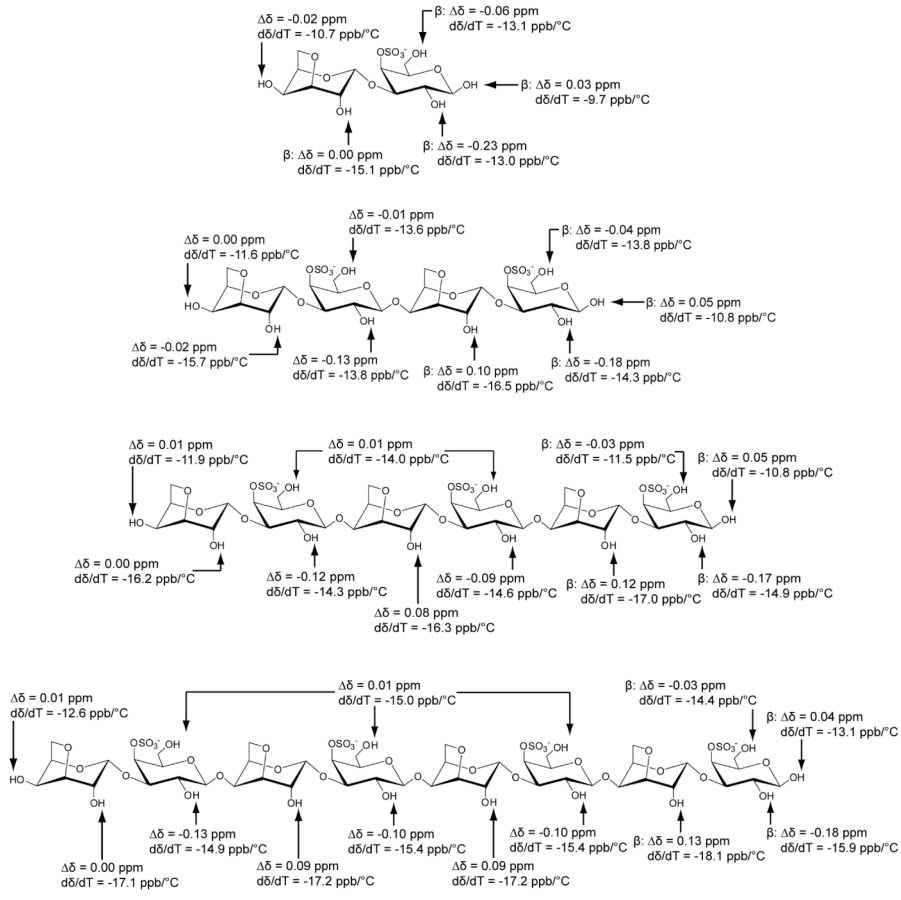

Figure 18. Δδ and dδ/dT for κ-carrageenan oligosaccharides **1**–**4**. Only data for the β-anomeric forms are shown.

In the κ/μ-oligosaccharides **5**–**7**, OH2_G4S at the reducing end and OH2_G4S(3) had negative Δδs (~ - 0.2 ppm), whereas those with one or two D6S residues as neighbors had Δδs close to zero. The OH2 and OH3 of D6S had consistently smaller |dδ/dT| than the other hydroxy protons (Figure 19). In the hexasaccharide κμκ, **5**, a crosspeak due to chemical exchange was observed between OH2_G4S(5) and OH3_D6S(4) in the ROESY spectra. In the octasaccharide κμμκ, **6**, chemical exchange between OH2_G4S(5) and OH3_D6S(4) and between OH2_G4S(7) and OH3_D6S(6) was observed (Figure 20).

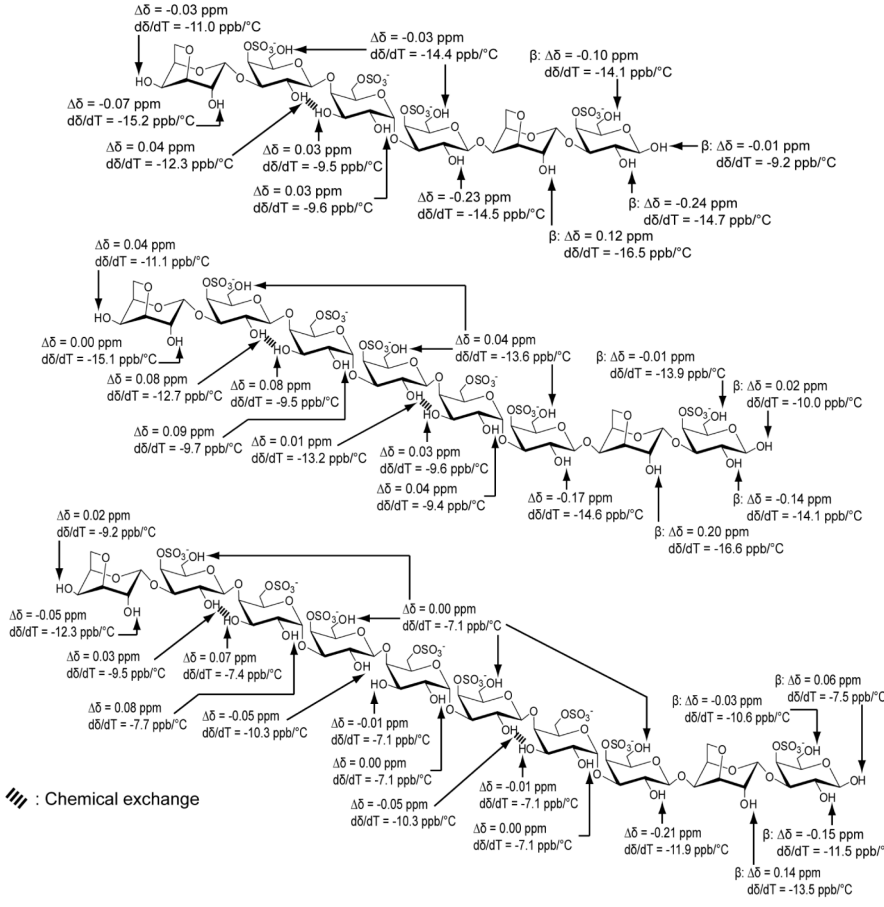

Figure 19. Δδ, dδ/dT and chemical exchange for hydroxy protons in κ/μ-carrageenan oligosaccharides **5**–**7**. Only data for the β-anomeric forms are shown (n.d.: not determined).

Comparison of the proton NMR spectra of κ and κ/μ hexa- and octasaccharides revealed significant downfield shifts of OH2_G4S(5) and (7) in κμκ and κμμκ compared to the corresponding protons in the κ-oligosaccharides (Figure 21). OH2_G4S(1) and (3) had on the other hand negative Δδs as in the κ-oligosaccharides. OH2_G4S(3) had a larger negative Δδ in the κ/μ sugars suggesting that a 3-linked D6S(4) residue reduces hydration of OH2_G4S(3) or that OH2_G4S(3) is in closer proximity to O5 of D6S(4) than O5 of DA.

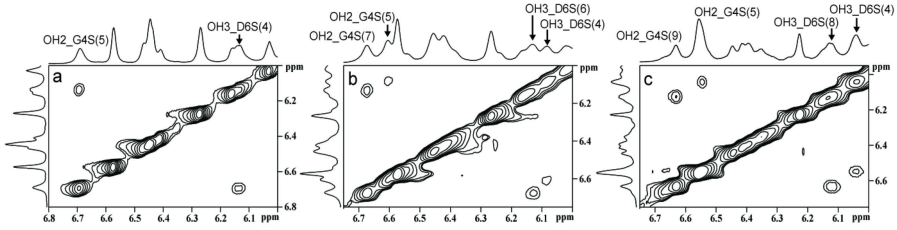

Figure 20. Part of the TOCSY spectra of (a) **5**, showing the chemical exchange crosspeaks between OH2_G4S(5) and OH3_D6S(4); (b) **6**, showing the chemical exchange crosspeaks between OH2_G4S(7) and OH3_D6S(6) as well as between OH2_G4S(5) and OH3_D6S(4); (c) **7**, showing two chemical exchange crosspeaks, one between the signals from OH2_G4S(9) and OH3_D6S(8) and the other one between the overlapping signals from OH2_G4S(5), (7) and OH3_D6S(4), (6).

The downfield shift of OH2_G4S(5) and (7) in compounds **5**–**6** compared to in compounds **3**–**4** is due to a different chemical surrounding with a D6S residue as its closest reducing-end neighbor instead of DA as in the κ-oligosaccharides. This together with the exchange crosspeaks discussed above suggests a transient hydrogen bonding interaction between OH2_G4S and OH3_D6S. The lower temperature coefficients of OH2 and OH3 of D6S can probably be explained by reduced interaction with water because of the spatial proximity to the 4-sulfate group and to the ring oxygen of G4S respectively. Such interactions have been predicted by MM3-calculations (Stortz, 2006), and these NMR data on hydroxy protons give the first experimental evidence that hydrogen bonding interactions can exist, at least transiently, in water solution.

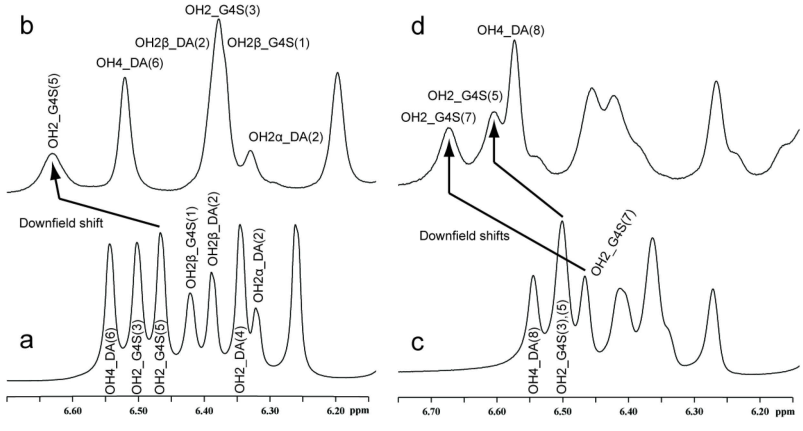

Figure 21. ^{1}H 1D NMR spectra (a) and (b) show the downfield shift of the OH2_G4S(5) signal in **5** (b) compared to in **3** (a). Spectra (c) and (d) show the downfield shift of the OH2_G4S(5) and OH2_G4S(7) signals in **6** (d) compared to in **4** (c).

There is one hydrogen bond in κμκ and two hydrogen bonds in κμμκ but comparison of the NMR data in the two compounds suggest that the interactions are not stronger in the larger oligosaccharide. For the decasaccharide κμμμκ, **7**, signal overlap precludes the unambiguous determination of whether three or only two of the OH2_G4S–OH3_D6S interactions are present. Examination of the crosspeak intensities in the TOCSY spectra suggested that only one of the pairs OH2_G4S(5), (7) and OH3_D6S(4), (6) have chemical exchange. If this is the case, the downfield shift observed for H1_D6S(6) could tentatively be attributed to the loss of a hydrogen bond and the subsequent increase in flexibility. On the other hand, the almost identical chemical shift and very similar temperature coefficients indicate similarities instead of differences in the structure. Faster exchange process in the larger κμμμκ oligosaccharide cannot be excluded. Different exchange processes in compounds **5**–**7** could mean that chemical exchange interactions between the OH2_G4S and OH3_D6S hydroxy protons might be present along all the oligosaccharide chain in κμμμκ as well.

Gelation is based on intermolecular hydrogen bonds in large polymers while the NMR data presented here only shows intramolecular hydrogen bonds in oligosaccharides. However, it cannot be excluded that what is observed at the oligosaccharide level is also present in the larger polymers. Thus, transient hydrogen bonding interactions in μ-carrageenan may be of importance for the macromolecular structuring properties of κ-carrageenan. Jouanneau *et al*. (2010) showed that the three main distributions of μ-carrabiose in the native κ-carrageenan were μ, μμ and μμμ. It is likely that most of the μ-carrabiose units in κ-carrageenan are excluded from helical regions and in turn from the junction zones formed upon gelling. The intrachain bonding found in μ-carrabiose may then implicate that the fractions between the junction zones do in fact possess a certain structure and might play a role in the organization of the κ-carrageenan gel network.

3.2 Paper II: Application of Diffusion-Edited NMR Spectroscopy for Selective Suppression of Water Signal in the Determination of Monomer Composition in Alginates

As described in the introduction, the physical properties of alginates such as gel properties and viscosity are largely correlated to the monomer composition (M/G-ratio), the block distribution and the molecular weight. Therefore, access to methods for accurate determination of the M/G-ratio is important and NMR spectroscopy is one of the most effective tools. Determination of the M/G-ratio, and also of the diad and triad composition, is done by integration of the

signals in the anomeric region of the ^{1}H spectrum. These signals are relatively well resolved and are characteristic for H1 and H5 of G and M residues in different surroundings as can be seen in figure 22.

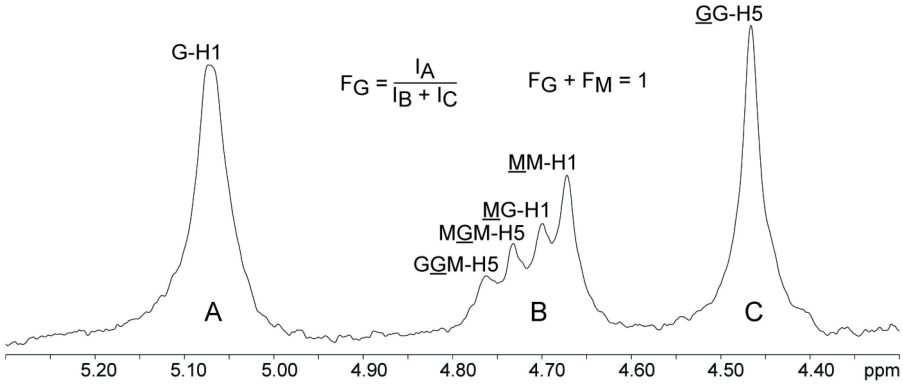

Figure 22. Anomeric region of a ^{1}H NMR spectrum of an alginate polysaccharide. Formula for calculation of monomeric fractions: F_M, fraction of M residues; F_G, fraction of G residues; I, integral. Assignments were taken from Salomonsen *et al*. (2009a).

Sometimes, however, the high viscosity of alginate samples does not allow the acquisition of highly resolved NMR spectra. A frequently used solution to this problem is to partially hydrolyze the alginate to lower the molecular weight and in turn the viscosity. This is however time consuming and it can also lead to sample alteration compared to the intact sample since different glycosidic linkages in alginate have different rates of hydrolysis (Salomonsen *et al.*, 2008; Holtan *et al.*, 2006; Grasdalen *et al.*, 1979b; Smidsrod *et al.*, 1969; Haug *et al.*, 1967a; Haug *et al.*, 1966; Smidsrod *et al.*, 1966). Different approaches have been proposed to overcome this problem including ^{1}H high-resolution magic angle spinning (HR-MAS) NMR of alginates suspended in D_2O and ^{13}C cross-polarization MAS (CP-MAS) NMR of alginate powders. Both these techniques have successfully been used to analyze the M/G-ratios of alginates (Salomonsen *et al.*, 2009b). Many laboratories are however not equipped with HR-MAS or CP-MAS probes. Additionally, temperatures of 80–90 °C have to be used to move the water signal away from the region containing the signals used for the M/G-ratio calculation and not all HR-MAS probes can be operated at such high temperatures. We have therefore investigated the possibility of using standard high resolution liquid NMR probes where the use of high temperatures does not pose a problem and thereby the water signal can, if needed, be moved away from the diagnostic signals.

In order to lower the sample viscosity the sample concentration is lowered which in turn makes the water signal become several orders of magnitude

larger than the alginate signals. This leads to baseline and dynamic range problems that may preclude a correct M/G-ratio determination. Therefore, water suppression is necessary. Commonly used water suppression techniques such as presaturation or those based on pulse field gradients lack selectivity and resonances that overlap with the solvent signal will also be suppressed. It has also been shown that water presaturation affect the signal intensity of the signals in the NMR spectra of alginates, leading to significant errors in M/G-ratio analysis (Salomonsen *et al.*, 2009a) (Figure 23).

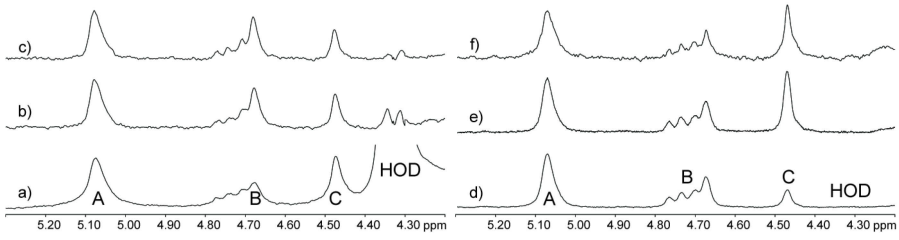

Figure 23. Comparison of different water suppression techniques applied on sample 01 (Table 1). (a) single pulse ^{1}H NMR spectrum, (b) presaturation (*zgpr*), (c) NOESY presaturation (*noesygppr1d*), (d) WATERGATE (*zggpw5*) with a too short pulse interval (*d*19 = 400 µs), (e) WATERGATE (*zggpw5*) with optimum pulse interval (*d*19 = 700 µs), (f) diffusion-edited (BPP-LED). All spectra were obtained at 70 °C.

An alternative approach is to use a diffusion-filtered NMR experiment that eliminates the water signal while retaining the signals of interest. This approach exploits the higher diffusivity of water molecules relative to the larger solute molecules.

The bipolar gradient pulse pair longitudinal eddy current delay pulse sequence (BPP-LED) (Wu *et al.*, 1995) was used (Figure 24). In this experiment the magnetization is kept longitudinal during the diffusion period and the eddy current delay, and the relaxation losses are dictated mainly by the longitudinal relaxation T_1 instead of T_2. In a first step, five alginate samples with representative M/G-ratios and viscosities were chosen from a set of 20 (Table 1), in order to establish sample preparation and data processing procedures as well as to evaluate different experimental temperatures. The diffusion parameters, Δ (diffusion delay) and δ (gradient pulse duration), were determined empirically since the molecular weights of the alginates were unknown and because of the large variation in sample viscosities.

For temperatures between 15 and 65 °C the water signal fully or partially overlapped with the signals between 4.4 and 5.2 ppm used for M/G-ratio determination.

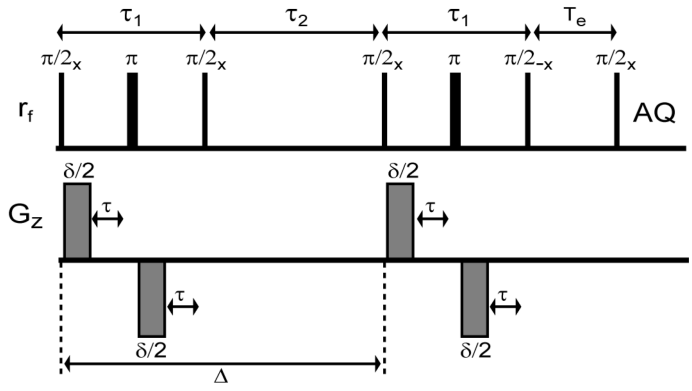

Figure 24. Schematic representation of the BPP-LED pulse sequence (Wu *et al.*, 1995). The figure is adapted from Dehner and Kessler (2005).

Accurate determination of M/G-ratios could be achieved for many samples at temperatures as low as 30 °C and at 50 °C it was possible for all types of alginate samples, regardless of high or low viscosity or high or low M/G-ratio (Figure 25).

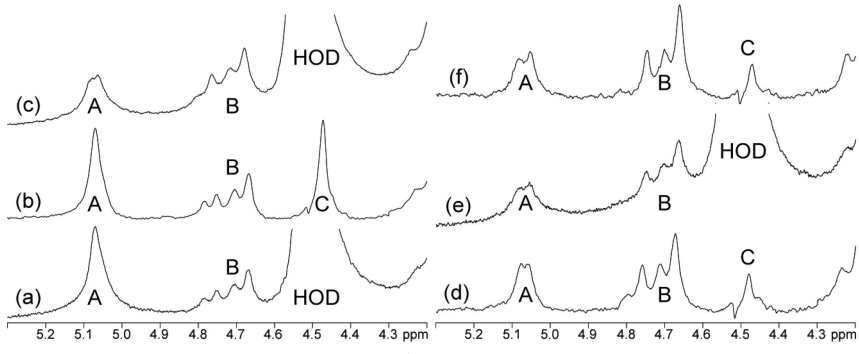

Figure 25. Anomeric region at 50 °C of a) ^{1}H NMR spectrum, sample 01, b) diffusion-edited spectrum sample 01, c) ^{1}H NMR spectrum, sample 17, d) diffusion-edited spectrum, sample 17, e) ^{1}H NMR spectrum sample 20, f) diffusion-edited spectrum, sample 20. Δ = 50 ms, δ = 2.0 ms.

For the development of a general protocol, a temperature of 70 °C was chosen to have the water signal upfield of the signals of interest for M/G-ratio calculation. Diffusion-edited spectra were obtained for 20 different alginate samples with varying M/G-ratios and viscosities (Table 1). Two sets of samples were recorded manually at 600 MHz and one set of samples was recorded at 400 MHz using a sample changer and the automation software ICON-NMR.

Table 1. *Values for M/G-ratios and viscosities. M/G-ratios[a] determined by FTIR. Viscosity[b] (mPa·s) determined on 1% solutions at 20 °C and 60 rpm using a Brookfield LVT. Sets 1, 2 and 3 are three separately prepared sample sets. The M/G-ratios were calculated from ^{1}H NMR spectra acquired at 70 °C with the BPP-LED pulse sequence using Δ = 50 ms and δ =1.3 ms. A minimum of 3 experiments per sample and sample set were run.*

Sample	M/G-ratio[a]	Viscosity [b]	Set 1		Set 2		Set 3		Sets 1,2 and 3	
			Avg M/G	SD	Avg M/G	SD	Avg M/G	SD	Avg M/G	SD
01	0.4	50	0.4	0.06	0.4	0.06	0.4	0.06	0.4	0.06
02	0.5	340	0.5	0.04	0.5	0.02	0.4	0.08	0.5	0.07
03	0.4	170	0.4	0.05	0.4	0.03	0.4	0.05	0.4	0.05
04	0.4	55	0.5	0.08	0.4	0.02	0.4	0.01	0.4	0.05
05	0.4	75	0.4	0.02	0.5	0.02	0.4	0.05	0.4	0.05
06	0.4	420	0.4	0.02	0.4	0.03	0.2	0.06	0.4	0.11
07	0.5	240	0.5	0.01	0.5	0.03	0.4	0.12	0.5	0.09
08	0.7	540	0.7	0.02	0.7	0.05	0.6	0.09	0.7	0.06
09	0.6	370	1.2	0.10	1.2	0.07	1.1	0.10	1.1	0.10
10	0.7	450	0.7	0.01	0.7	0.02	0.6	0.12	0.6	0.06
11	0.7	270	0.7	0.03	0.7	0.04	0.6	0.02	0.7	0.04
12	0.9	390	0.8	0.05	0.8	0.04	0.7	0.16	0.8	0.08
13	0.9	360	0.9	0.01	0.9	0.02	0.8	0.08	0.9	0.06
14	1.2	760	1.1	0.05	1.0	0.03	1.0	0.19	1.0	0.13
15	1.3	9	0.9	0.06	1.2	0.01	1.1	0.07	1.1	0.12
16	1.3	500	1.3	0.01	1.3	0.03	1.1	0.08	1.2	0.10
17	1.5	64	1.6	0.06	1.6	0.11	1.4	0.05	1.5	0.11
18	1.5	1400	1.1	0.07	1.1	0.05	1.0	0.12	1.0	0.09
19	1.8	420	2.1	0.07	2.1	0.05	1.8	0.08	2.0	0.15
20	1.7	1140	1.7	0.10	1.9	0.11	1.5	0.18	1.7	0.20

The M/G-ratios were in good agreement with those obtained by FTIR (Table 1). The standard deviations (SD) within triplicates and between sample sets were generally low, below 0.1. Previously published SDs determined on commercially available alginates (Salomonsen *et al.*, 2008) correlate well with the SDs for M/G-ratios of sample sets 1 and 2 in table 1. For samples with extreme values more specific diffusion parameters can be used.

Diffusion-edited NMR as a solvent suppression technique is not limited to alginates but is also useful for studying other biopolymers. It was for example found very useful for selectively removing several interfering signals from buffer (Paper III) to enable interpretation of sample signals. Since diffusion-edited NMR can be used for water suppression in the analysis of alginates at

temperatures below 50 °C, it permits the use of probes that cannot be used at high temperatures such as cryoprobes or certain HR-MAS probes.

3.3 Paper III: The First Bacterial Glycosaminoglycan Endosulfatase Reveals Novel Metabolic Pathways in the Prominent Human Gut Symbiont *Bacteroides thetaiotaomicron*

To identify novel sulfatases that would modify the sulfation pattern of GAGs with the goal of producing new sulfated oligosaccharides with new therapeutic properties, *Bacteroides thetaiotaomicron*, a GAG degrading bacteria, present in the human gut was investigated. Eleven sulfatases induced in presence of host glycans were cloned, expressed and tested for sulfatase activity. As a first step in the biochemical characterization of the enzymes, the activity as a generic arylsulfatase was assayed using the chromogenic *p*NP-S as a substrate. Only a few of the eleven enzymes were found to be active against this substrate (Paper III, supplementary information, Table 1). Four of the eleven *in vivo* induced enzymes were induced *in vitro* in presence of GAGs (Benjdia *et al.*, 2011; Martens *et al.*, 2008). Two of the *in vitro* induced enzymes, BT1596 and BT3349, hydrolyzed *p*NP-S making them authentic sulfatases while two others, BT4656 and BT3333, were not. The activity of the four enzymes was further tested towards a broad variety of sulfated substrates using various analytical techniques. These substrates were polymeric GAGs such as heparin and chondroitin (Figure 9), a synthetic library of chondroitin disaccharides with various sulfation patterns (Figure 26), mixtures of unsaturated disaccharides obtained by the action of lyases on CS and HS polymers as well as monomeric substrates and saturated and unsaturated oligosaccharides (Figure 33).

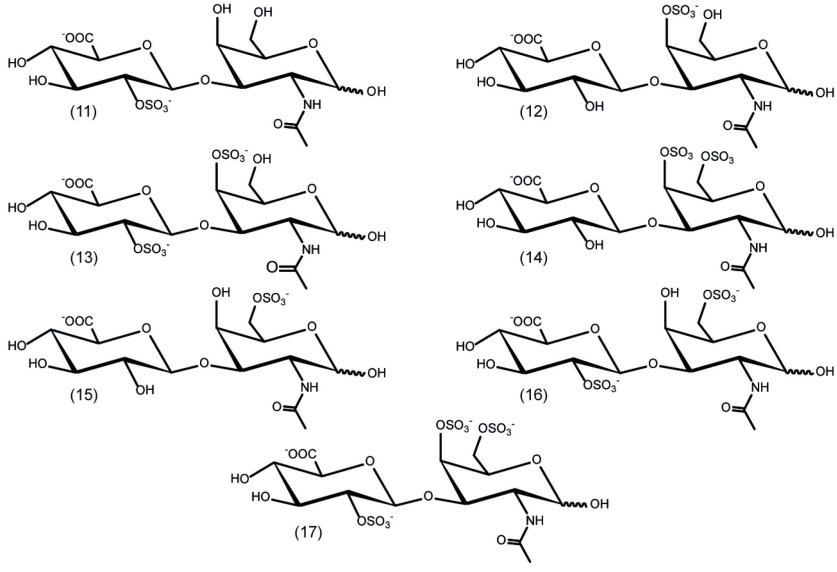

Figure 26. Structures of the CS disaccharides **11**–**17** in the synthetic library (Lubineau & Bonnaffe, 1999) used for testing sulfatase activity.

Sulfatase BT3349 identified as a bacterial endo-4-O-sulfatase

When incubated with polymeric shark chondroitin BT3349 was the only enzyme capable of removing sulfate from CS. Analysis of the 2D COSY, TOCSY and HSQC NMR spectra of the polymer before and after addition of BT3349 clearly showed that upon addition of the enzyme all the signals corresponding to H4 of GalNAc sulfated at the C4 position were suppressed demonstrating that the enzyme was hydrolyzing all 4-sulfate groups of the GalNAc4S units in the polymer chain (Figure 27).

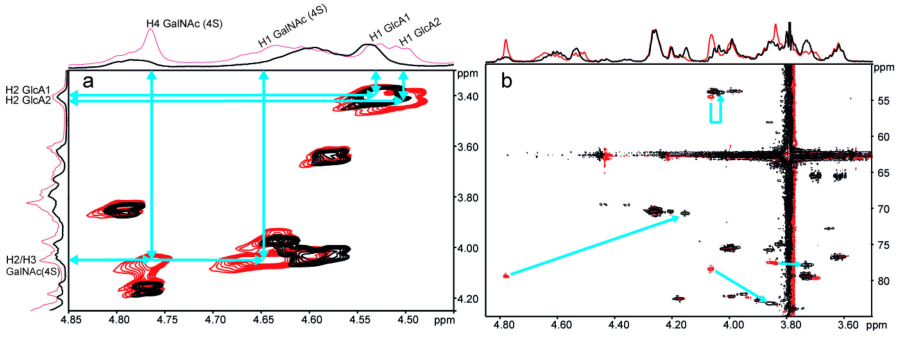

Figure 27. 2D TOCSY (a) and HSQC spectra (b) comparing polymeric CS before (red) and after (black) incubation with BT3349. In the HSQC spectrum the shifts of signals from H2/C2, H3/C3, H4/C4 and H5/C5 are visualized by the light blue arrows.

BT3349 desulfated not only CSA, mainly monosulfated at C-4 of GalNAc but also other types of CS chains such as CSB, CSD and CSE with various degrees and positions of sulfation (Figure 4A, B Paper III).

The extent of desulfation varied for the different substrates, depending on the sulfation pattern. Mono 4-*O*-sulfated chains were essentially completely hydrolyzed but sulfation at positions other than C-4 as well as epimerization of the hexuronate unit affected the accessibility of the enzyme (Figure 4A and C in Paper III). As distribution of sulfate groups in different chain types is heterogeneous, analysis at chain level gives a semi-quantitative indication of the impact of modification surrounding the target sulfate group.

Screening of BT3349 towards a library of chondroitin disaccharides (Figure 26) showed that disaccharides with sulfate groups in the 4-position of GalNAc were also substrates for the enzyme. Comparison of the NMR spectra of the disaccharide GlcA-GalNAc4S6S (Figure 26, compound **14**) before and after addition of BT3349 showed that the signals at 4.8 and 4.9 ppm corresponding to H4-GalNAc4S6S disappeared, demonstrating desulfation at position 4 of GalNAc and thus confirming the high specificity for the C-4 position (Figure 28).

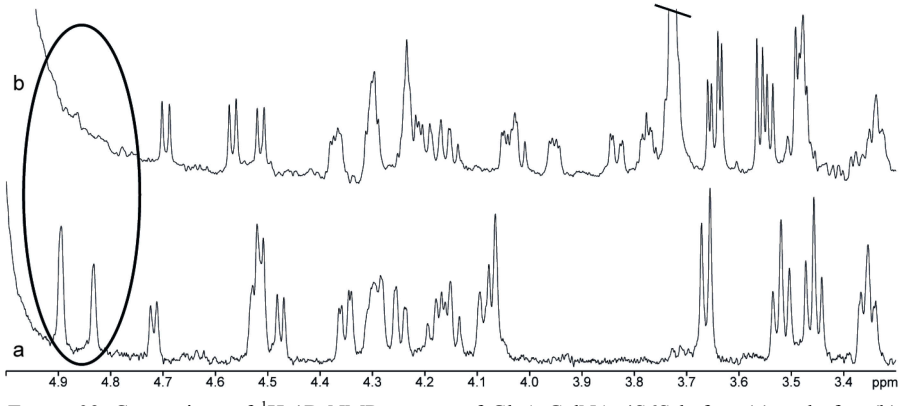

Figure 28. Comparison of ^{1}H 1D NMR spectra of GlcA-GalNAc4S6S before (a) and after (b) incubation with BT3349. The characteristic signals of H4(α/β) of GalNAc4S6S at 4.9 and 4.8 ppm disappear upon incubation with the enzyme.

4,5-Unsaturated hexuronate (Δ) disaccharides obtained from the different types of CS by hydrolysis with lyases were also substrates for BT3349. They were therefore further used to determine the influence of sulfate groups in position C-2 of the hexuronate sugar and position C-6 of the galactosamine on the efficiency of the enzyme. The results showed that GalNAc 6-*O*-sulfation only slightly affects the activity, whereas Δ 2-*O*-sulfation hampered BT3349

activity significantly. Sulfation at all three possible positions rendered the substrate even less accessible to the enzyme (Figure 4D, Paper III).

Identification of two 6-O-sulfatases

Since BT3333 was found to be inactive towards polymeric CS (Figure 29) and HS as well as towards both saturated and unsaturated disaccharides an exolytic mechanism of the enzyme was suspected.

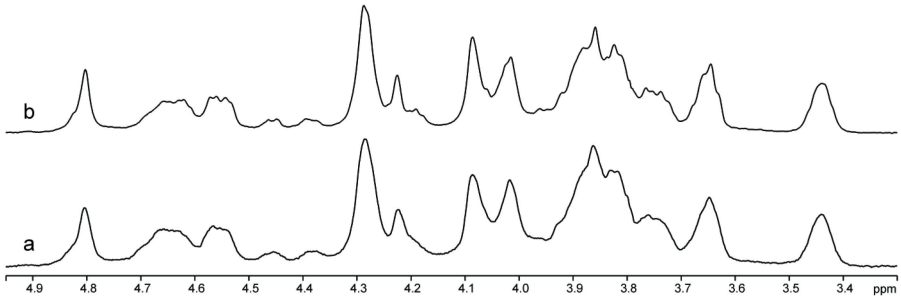

Figure 29. ^{1}H 1D NMR spectra of polymeric shark CS alone (a) and incubated with BT3333 (b).

BT3333 was found to be active towards GalNAc-6-*O*-sulfate but not towards GlcNAc-6-*O*-sulfate making the enzyme a strict exolytic *N*-acetylgalactosamine-6-*O*-sulfatase (Figure 2B, Paper III).

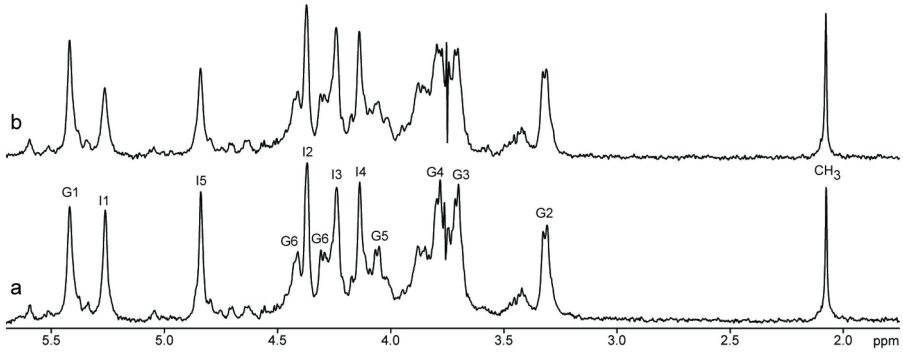

Figure 30. Diffusion-edited NMR spectra of heparin alone (a) and incubated with BT4656 (b). G denotes protons from GlcNAc and I from IdoA. Intensity differences for IdoA signals I1 and I5 can be corrected by addition of EDTA to the incubated sample.

BT4656 was found to be inactive against heparin/HS (Figure 30) and CS polymeric substrates. BT4656 was also assayed for sulfatase activity towards HS disaccharide substrates with either a hexuronate or a 4,5-unsaturated hexuronate residue in the non-reducing end but no activity was found. The

enzyme was assigned 6-*O*-sulfatase activity from a UV-based assay involving the chromogenic substrate 6-*O*-sulfo-GlcNAc-*O*-*p*NP. The assay was designed so that BT4656, 6-*O*-sulfo-GlcNAc-*O*-*p*NP and β-*N*-acetylglucosaminidase were incubated together and the release of *p*NP was monitored at 405 nm. β-*N*-acetylglucosaminidase is only able to cleave the *p*NP moiety if the 6-*O*-sulfate group has been hydrolyzed. The 6-*O*-sulfatase activity and role of BT4656 as an exolytic *N*-acetylglucosamine-6-*O*-sulfatase was corroborated by NMR analysis (Figure 31).

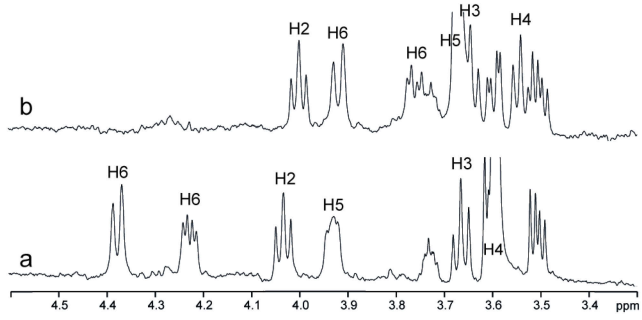

Figure 31. Parts of ^{1}H 1D NMR spectra of 6-*O*-sulfo-GlcNAc-*O*-*p*NP in presence of BT4656 before (a) and after (b) desulfation. Action of the enzyme results in an upfield shift of the H6 signals, indicative of desulfation. Unassigned signals are from buffer.

In an attempt to identify the interactions between BT4656 and this substrate, STD NMR experiments were performed. Large STD signals, evidence of binding, were observed (Figure 32).

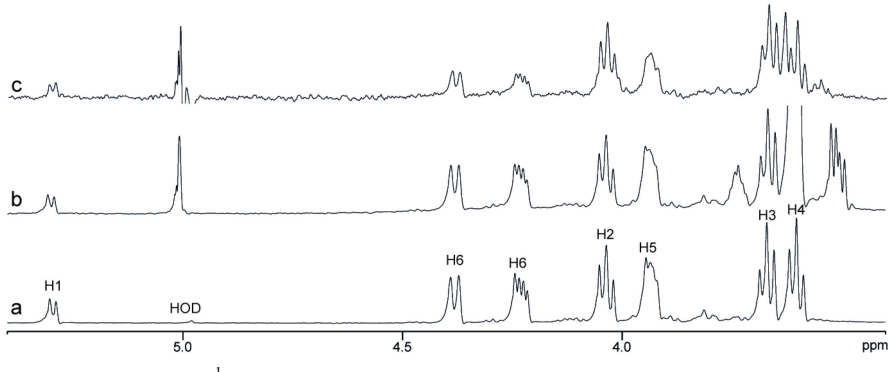

Figure 32. Part of the ^{1}H NMR spectra of 6-*O*-sulfo-GlcNAc-*O*-*p*NP alone (a), incubated with BT4656 (b) and part of STD spectrum of 6-*O*-sulfo-GlcNAc-*O*-*p*NP incubated with BT4656 (c).

The largest enhancement was seen for H4, H2, H3 and the *N*-acetyl group. H5 and H6 showed the weakest STD effects. STDs to the phenyl protons of *p*NP

were also observed. The buffer did not bind the protein and consequently no signals from the corresponding protons were observed in the STD spectra indicating that the effects observed were due to true saturation transfer.

Identification of an exolytic 2-O-sulfatase

BT1596 showed no activity against polymeric HS. The activity of BT1596 was then investigated by incubating the enzyme with a heparin hexasaccharide (Petitou *et al.*, 1988) as well as with the hemisynthetic ultra-low molecular weight heparin AVE5026 (Figure 33). AVE5026 has an average MW of around 2400 Da and with a degree of sulfation being about 2.0 per disaccharide unit (Viskov *et al.*, 2009). In the hexasaccharide, only Δ is sulfated at the 2-*O*-position while in AVE5026 both Δ and the internal IdoA residues are partially 2-*O*-sulfated (Figure 33).

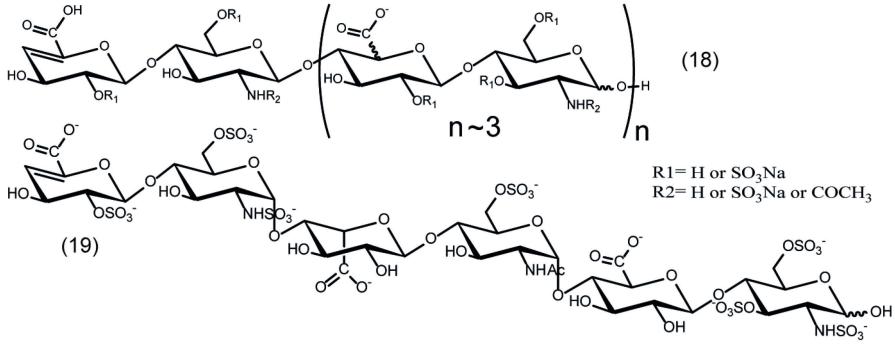

Figure 33. Schematic structures of AVE5026 (**18**, top) and hexasaccharide (**19**, bottom). Compounds are present as Na^+-salts.

Comparison of the ^1H and ^{13}C signals for the hexasaccharide before and after incubation with BT1596 showed chemical shift differences for the signals H1/C1 to H4/C4 at the non-reducing end (Figure 34). The large upfield shifts of H2 (0.84 ppm) from 4.64 ppm to 3.80 ppm together with the carbon upfield shift of circa 4 ppm (not shown) proved desulfation at this position. The changes in chemical shifts for the other signals are also characteristic of desulfation at the 2-position (Guerrini *et al.*, 2002; Pervin *et al.*, 1995).

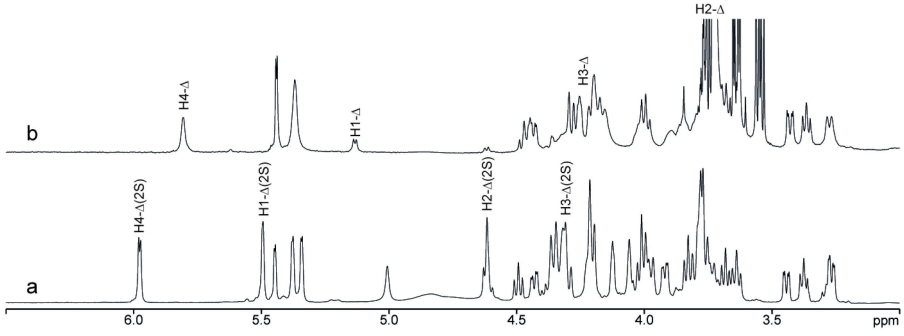

Figure 34. [1]H 1D NMR spectra of hexasaccharide (**19**) before (a) and after (b) incubation with BT1596.

To confirm the exolytic character of the enzyme its activity was tested towards AVE5026. NMR analysis of the AVE5026 oligosaccharides before and after incubation with BT1596 demonstrated that the enzyme is exclusively hydrolyzing the sulfate ester from the 2-position of the non-reducing end of all oligosaccharides present in AVE5026 making it a Δ-4-hexuronate-2-*O*-sulfatase (Figure 35).

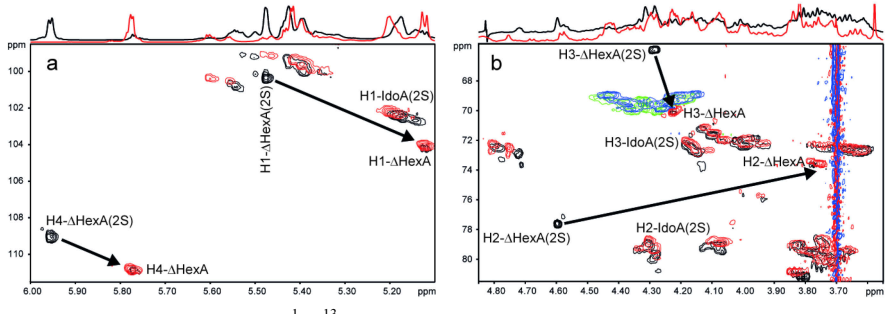

Figure 35. Superimposition of [1]H-[13]C HSQC spectra of AVE5026 alone (black and green) and incubated with BT1596 (red and blue) showing the large shifts experienced by the carbons and protons of the non-reducing end residue upon desulfation at position 2. The spectra also show that internal IdoA2S remains sulfated. Spectrum (a) displays the anomeric region and spectrum (b) the H2 to H6 region.

The desulfation is specific for the non-reducing end since the signals for internal IdoA2S did not show any significant changes in chemical shift (Figure 35 and 36). A desulfation at C2 of IdoA2S would have resulted in a chemical shift change for the corresponding H2 proton from 4.32 ppm to 3.8 ppm (Pervin *et al.*, 1995). Further the intensity of the H1-IdoA2S signal was the same before and after enzyme addition indicating no desulfation. BT1596

desulfated at the 2-position on the non-reducing end, invariably of the oligosaccharide size.

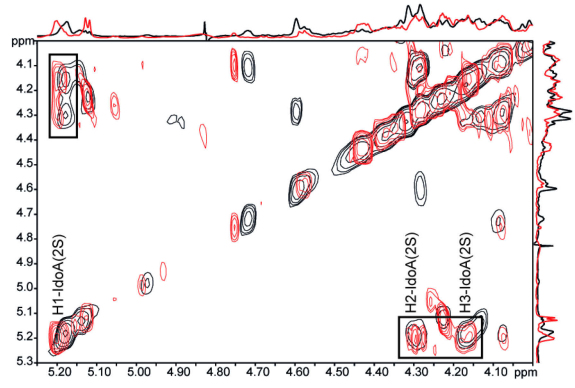

Figure 36. Superimposition of part of TOCSY spectra of AVE5026 alone (black) and incubated with BT1596 (red). No significant changes are observed in chemical shifts of internal IdoA2S H2 and H3.

BT1596 was also tested for sulfatase activity against an array of unsaturated HS and CS disaccharides. All types of 2-*O*-sulfated compounds were substrate for the enzyme independently of their hexosamine unit and additional sulfation (Figure 2B, Paper III). To verify that 4,5-unsaturation of the non-reducing end hexuronate was required for desulfation BT1596 was incubated with a saturated synthetic octasaccharide (Figure 37) and analyzed by NMR. No signs of desulfation were observed (Paper III, supplementary information, Figure 5).

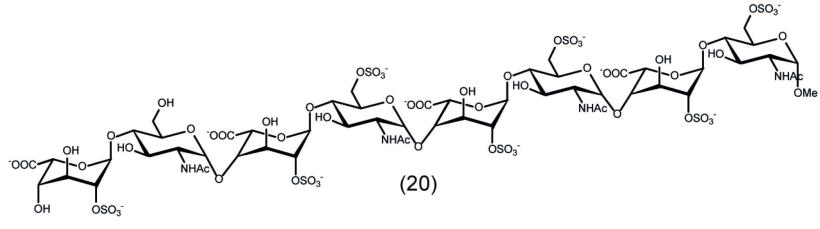

Figure 37. Schematic structure of the synthetic octasaccharide (**20**), the compound is a Na^+-salt.

In view of these results a degradation pathway of GAGs by sulfatases from *B. thetaiotaomicron* could be tentatively proposed (Figure 6 in Paper III). *B. thetaiotaomicron* has the possibility to desulfate polymeric CS on the 4-position before degradation by GAG lyases into oligosaccharides and subsequently further desulfation and degradation can take place. Polymeric heparin/HS on the other hand are first degraded into oligosaccharides that are then desulfated, further degraded and finally additional desulfation occur.

3.4 Paper IV: NMR Study on the Interaction of Trehalose With Lactose and its Effect on the Hydrogen Bond Interaction in Lactose

Disaccharides like sucrose and trehalose are known for protecting biomolecules such as proteins and membranes from freezing, heating, desiccation and osmotic shock (Sampedro *et al.*, 2001; Sampedro *et al.*, 1998; Iwahashi *et al.*, 1995; Leslie *et al.*, 1995; Hottiger *et al.*, 1994). Four hypotheses, not necessarily mutually exclusive, have been proposed to explain the stabilizing effect of trehalose: (1) the water replacement hypothesis (Allison *et al.*, 1999), (2) the water entrapment or preferential hydration hypothesis (Belton & Gil, 1994), (3) the high viscosity hypothesis (Sampedro & Uribe, 2004; Green & Angell, 1989) and (4) the water destructuring effect mechanism (Lerbret *et al.*, 2005a; Lerbret *et al.*, 2005b; Branca *et al.*, 1999a; Branca *et al.*, 1999b).

Since the bioprotective properties of the sugars are thought to involve the hydroxyl groups and their interactions with water, it was investigated if and how the hydration and hydrogen bonding interaction in lactose (Figure 3) was affected by addition of trehalose or sucrose. For this the chemical shifts (δ), chemical shift differences (Δδ), coupling constants, ($^3J_{CH,OH}$), temperature coefficients (dδ/dT) and rotating frame nuclear Overhauser effects (ROE) of the hydroxy protons of lactose alone in solution and in the presence of trehalose or sucrose were measured over a large range of concentrations. The NMR data for trehalose and sucrose alone in solution were also collected. The experiments were performed in a 90/10% $H_2O/(CD_3)_2CO$ solvent mixture. The diffusion of water, lactose and trehalose in binary and ternary systems was also investigated over a large range of concentrations and temperatures using DOSY.

In water/trehalose and water/lactose binary systems the sugars have similar diffusion properties at low temperatures (< 40 °C), while at high temperatures and high concentrations the lactose diffusion rate was slightly higher. At low sugar concentrations water had the same diffusion in both systems and over the entire range of temperatures while for high sugar concentration the diffusion rate of water was lower with trehalose for temperatures above 45 °C, in good agreement with previous results (Ekdawi-Sever *et al.*, 2003; Iannilli *et al.*, 2001; Rampp *et al.*, 2000; Magazu *et al.*, 1999). In water/trehalose/lactose ternary systems both sugars had similar diffusion properties at all temperatures and concentrations investigated.

The presence of a weak hydrogen bond between OH3 in glucose (GlcOH3) and O5' in galactose (GalO5') in supercooled lactose has been deduced from the decrease in the chemical exchange rate of GlcOH3 as well as from the

small value of its $^{3}J_{CH,OH}$ (3.1 Hz) (Poppe & Vanhalbeek, 1994). For sucrose, under supercooled conditions, the existence of a transient hydrogen bond between the GlcOH2 and FruOH1' groups was evidenced from the chemical exchange observed between GlcOH2 and FruOH1' (Sheng & Vanhalbeek, 1995). For trehalose, the $^{3}J_{CH,OH}$ and $^{2,3}J_{C,OH}$ of the hydroxy protons, measured in 97/3% H_2O/D_2O solvent at −3 °C, suggested that no intramolecular hydrogen bonding interactions were present (Batta & Kover, 1999).

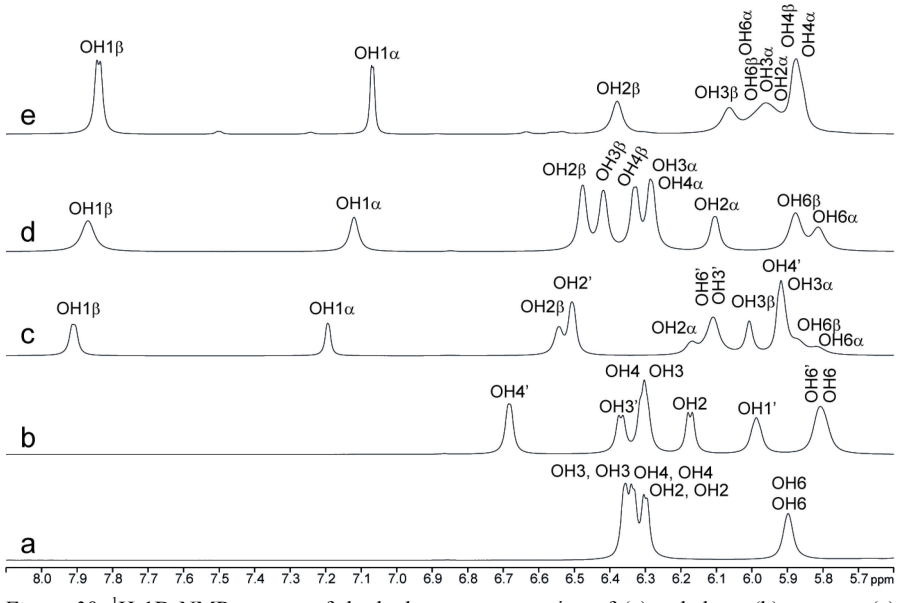

Figure 38. ^{1}H 1D NMR spectra of the hydroxy proton region of (a) trehalose, (b) sucrose, (c) lactose, (d) glucose and (e) galactose at −5 °C in 90/10% $H_2O/(CD_3)_2CO$. Notice the large upfield shifts of GlcOH3(α/β) in spectrum (c) compared to spectrum (d).

The data obtained in this work for lactose, trehalose and sucrose were in good agreement with those previous studies. The hydroxy protons in lactose had small |Δδ| (< 0.2 ppm) with the exception of GlcOH3(α, β) which had |Δδ| of 0.4 ppm (Figure 38) indicating spatial proximity to GalO5' and in agreement with the hydrogen bond between GlcOH3 and GalO5'. In trehalose, a Δδ of +0.21 for OH2 suggested proximity to another hydroxy group. According to previous simulation studies this might reflect an inter-residue, direct or water mediated, interaction with OH6 or a water bridged interaction between the two OH2 groups across the glycosidic linkage (Verde & Campen, 2011; Nunes *et al.*, 2010; Engelsen & Pérez, 2000; Conrad & de Pablo, 1999; Liu *et al.*, 1997). In sucrose the |Δδ| values were below 0.06 ppm.

In trehalose chemical exchange between OH3/4 and OH6 as well as between OH2 and OH6 were observed (Figure 39). Due to the symmetry of the molecule it was not possible to discriminate between intra- or interresidual interactions. According to simulation data from literature OH3/4–OH6 interactions can occur both intra- and inter-residually while OH2–OH6 interaction only occurs inter-residually (Sapir & Harries, 2011; Conrad & de Pablo, 1999; Liu *et al.*, 1997). In lactose only weak intraresidual exchange crosspeaks within galactose were observed (Figure 39).

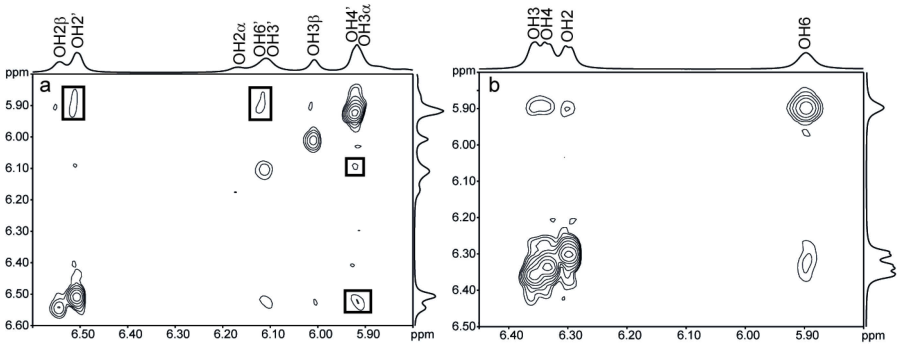

Figure 39. ROESY ^{1}H NMR spectra at –5 °C of (a) lactose 203 mg/mL and (b) trehalose 206 mg/mL.

In sucrose chemical exchange of hydroxy protons was observed between GlcOH2 and FruOH1' (Figure 40). In addition, a weak exchange was also observed between GlcOH2 and FruOH3' at high sugar concentration. The existence of both interactions at high concentration implies that two interconverting conformers exist in solution (Davies & Christofides, 1987).

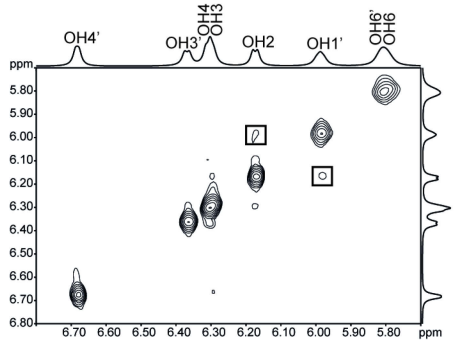

Figure 40. ROESY ^{1}H NMR spectrum at –5 °C of sucrose 202 mg/mL, mixing time 80 ms.

In the trehalose/lactose/water ternary system the temperature coefficients decreased by ≤ 1ppb/°C when increasing the sugar concentrations. Similar

effects were also measured in the binary systems. The $^{3}J_{CH,OH}$ did not change significantly except for GlcOH3 β, which decreased from 3.5 to 2.8 Hz. Small upfield shifts ($\Delta\delta_{mix}$) for the hydroxy protons of both lactose and trehalose were observed. The $|\Delta\delta_{mix}|$ increased as the sugar concentrations increased. The chemical shifts of the GlcOH3α/β signals were however significantly less affected by changes in concentration as reflected by their smaller $|\Delta\delta_{mix}|$ (Figure 41).

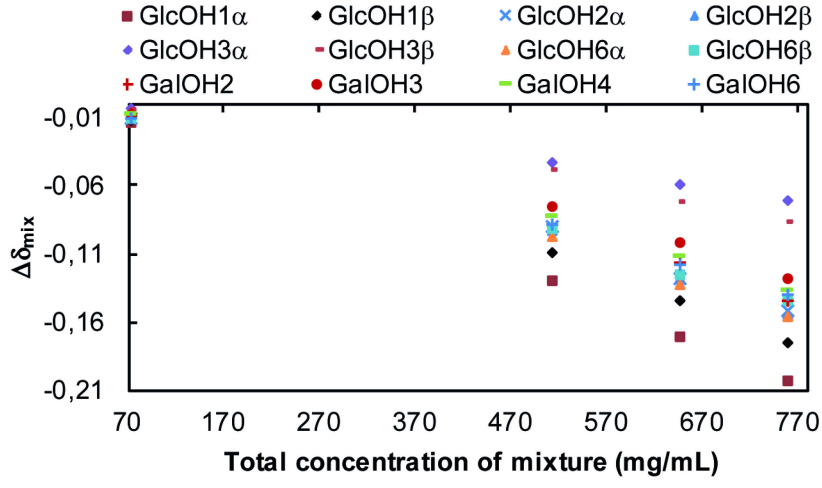

Figure 41. Graphical representation of $\Delta\delta_{mix}$ values. Δδmix: δ(Trehalose/Lactose X + X mg/mL) – δ(Trehalose/Lactose 37 + 36 mg/mL), except for the 37 + 36 mg/mL mixture where Δδmix: δ(Trehalose/Lactose 37+36 mg/mL) – δ(Lactose 35 mg/mL)

Chemical exchange was observed between hydroxy protons of trehalose and lactose, (Figure 42). GlcOH3 in lactose display less chemical exchange with trehalose. To determine whether the effects measured on lactose hydroxy protons were specific for interaction with trehalose the sucrose/lactose/water ternary system was analyzed under analogous conditions.

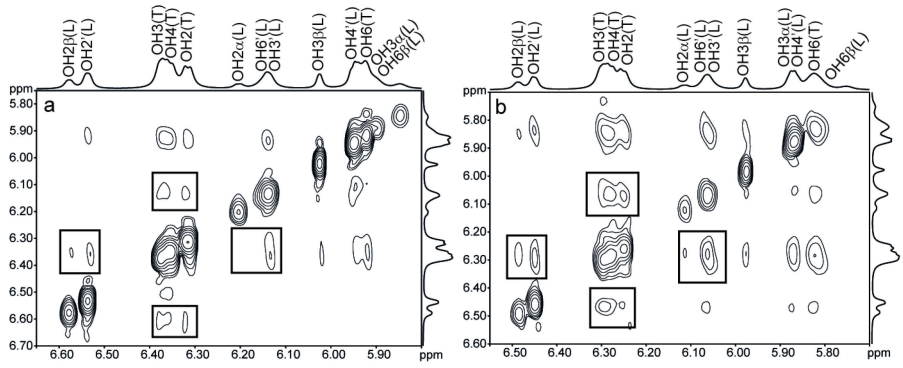

Figure 42. ROESY ^{1}H NMR spectra at –5 °C of (a) trehalose/lactose 37/36 mg/mL mixture, mixing time 80 ms and (b) trehalose/lactose mixture 256/256 mg/mL, mixing time 100 ms.

Very similar results were obtained for the coupling constants, temperature coefficients and $\Delta\delta_{mix}$. In the ROESY spectra, the intramolecular chemical exchange crosspeak between GlcOH2 and FruOH1' in sucrose had the strongest intensity while only weak chemical exchange crosspeaks between sucrose and lactose were observed (Figure 43).

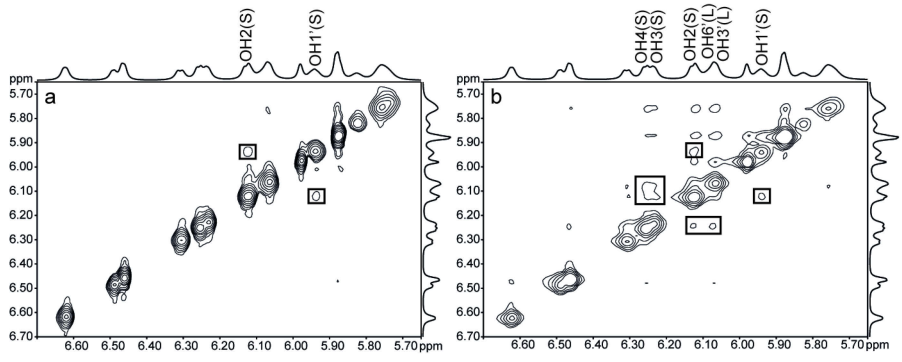

Figure 43. ROESY ^{1}H NMR spectra at –5 °C of (a) sucrose/lactose 178/181 mg/mL mixture, mixing time 40 ms and (b) sucrose/lactose mixture 178/181 mg/mL, mixing time 80 ms.

GlcOH3 of lactose was slightly less affected by the addition of trehalose or sucrose, attributed to its spatial proximity to GalO5' which restricts interaction with the surroundings. The data suggested that trehalose and sucrose did not significantly or differently affect the hydrogen bonding in lactose in the concentration range investigated.

The general upfield shift of hydroxy proton signals observed when increasing the sugar concentrations was attributed to reduced hydration due to slowed down water dynamics as shown in simulations with trehalose and proteins (Corradini *et al.*, 2013; Lins *et al.*, 2004). In support of these results is

the recent study by Lupi *et al*. who proposed that the concentration of hydroxy groups rather than the type of sugar is important for sugar-sugar and sugar-water interactions (Lupi *et al.*, 2012), at least for sugar concentrations ≤ 50% (w/w). It has also been shown that at low concentrations the hydration of trehalose is similar to that of other organic solutes (Winther *et al.*, 2012).

At concentrations above 40% (w/w) where water retardation in sugar solutions is strongly increased and differentiation between sugars start (Lerbret *et al.*, 2011), the effect of trehalose and sucrose on the hydration and hydrogen bonding in lactose might be differentiated. However this could not be investigated due to the low temperatures required for NMR studies of hydroxy protons.

4 Concluding Remarks and Further Perspectives

The work presented in this thesis was aimed at the analysis of carbohydrate structures, properties and interactions using NMR spectroscopy. Much of the work was done within the PolyModE project and was focused on carrageenans, alginates and GAGs.

In paper I the structures of κ- and κ/μ-hybrid carrageenan oligosaccharides were investigated using NMR of hydroxy protons. It was found that in κμκ and κμμκ there is an interresidual hydrogen bond interaction between OH2_G4S and OH3_D6S across the 1→4 linkage of the μ-carrabiose units. In the κμμμκ decasaccharide the NMR data suggested that this hydrogen bond is lost in the central μ-carrabiose unit. It cannot be excluded that what is observed at the oligosaccharide level is also present in the larger polymers. The existence of hydrogen bonds in κ/μ-carrageenan and hence a certain structural rigidity may infer that the μ-carrabiose units, mostly found in the non-helicoidal regions of the κ-carrageenan gel network have an underestimated role in the structural organization of the κ-carrageenan gel network. Studies of larger κ/μ-hybrid structures that more closely resemble polymeric structures would be interesting and could yield new information important for describing the structural implications of hybridity in carrageenans

In paper II a method for measuring the monomeric composition in intact alginate samples in solution was developed, using diffusion-edited NMR. Minimal sample preparation was required and the method was applicable to the screening of large sample sets. The experiment could also be employed to determine the M/G-ratio of intact alginate samples at temperatures below 50 °C allowing the utilization of NMR probes that cannot be used at high temperature, such as cryoprobes and certain HR-MAS probes. Diffusion-edited NMR experiments were also shown to be highly applicable to studies of biomolecules where buffer components and solvents produced NMR signals

that overlapped and obscured the signals of interest. The use of presaturation methods for water suppression will lead to an increase in intensity of the signals from M-residues in alginate. We have made similar observations with hyaluronic acid and other polysaccharides with large water retention capabilities. In the case of alginates the increase in signal intensities has been attributed to closer water association in the M-blocks due to a more linear and flexible structure (Salomonsen *et al.*, 2009a). Further investigation of the effect of presaturation of water on the NMR spectra of these types of polymers could help to understand, at an atomic level, how the polymers interact with water.

In paper III four novel GAG-sulfatases from the human gut commensal *Bacteroides thetaiotaomicron* were identified and their specificity determined. The enzyme BT3349 was found to be the first bacterial GAG endolytic-*O*-sulfatase and was able to remove sulfate groups at the 4-position of *N*-acetylgalactosamine from disaccharides to polymers of CS/DS. The other three enzymes were strictly exolytic. Two of them, BT3333 and BT4656, were 6-*O*-sulfatases specific for *N*-acetylgalactosamine and *N*-acetylglucosamine sugars respectively, thus participating in the degradation of CS/DS and HS. The fourth enzyme, BT1596, a Δ-4-hexuronate-2-*O*-sulfatase, active on HS and CS/DS oligo- and disaccharides produced from their respective polymers by lyase activity. The complementary set of sulfatase enzymes present in *Bacteroides thetaiotaomicron* enlighten that GAGs are likely metabolized by different, non-parallel pathways with common steps. Further characterization of the GAG degradation pathways of *Bacteroides thetaiotaomicron* could not only provide us with knowledge about the GAG metabolism of bacteria but also provide us with enzymatic tools for the production of tailored GAG structures.

In the fourth paper it was shown that trehalose and sucrose had small and similar effects on the hydration and hydrogen bonding interaction of lactose. The results suggested that it is the concentration of hydroxy groups that influences the sugar-sugar and sugar-water interactions at concentrations below 40% (w/w), more than the type of sugar.

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