Improved characterization techniques for branched polysaccharides

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“I am not young enough to know everything.”
Oscar Wilde

I would like to dedicate this book to my parents and grandparents.

Contents

CHAPTER 1

GENERAL INTRODUCTION

Starch, glycogen and cellulose are all around us. We eat them and use them on a daily basis but we do not understand them completely. Even though these polysaccharides are simple concerning their repeating unit they are hard to characterize. In order to try to understand as much as possible about their structure and the relationship between molecular structure and the physical properties, it is very practical to create this type of polysaccharides, for instance enzymatically, characterize and use them as standards for the characterization of natural ones. Therefore, the main objective of this thesis is centered on different enzymatic routes to this type of polysaccharides, possibilities for their characterization and the characterization of natural ones.

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1.1 Sugar-licious. The most abundant polysaccharides in nature

The most abundant organic substances on this planet are saccharides ($C_n(H_2O)_m$), also known as polysaccharides or sugars. Depending on the structure saccharides can be divided into mono-, di- or polysaccharides, where monosaccharide is the simplest one and cannot be hydrolyzed to smaller saccharides. Monosaccharides can either be aldoses or ketoses, depending on the group, which they contain: aldehyde or ketone, respectively. The smallest aldose (glyceraldehyde) possesses 3 carbons, out of which one is a stereo-center and therefore glyceraldehyde exists as a pair of enantiomers, L and D. L/D-glyceraldehyde serve as the reference for the further configuration of saccharides.
Glucose is one of the simple saccharides, nevertheless the most important among prokaryotes and eukaryotes when it comes to the source of energy used for metabolic processes. Glucose is a hexose, and has 4 stereo-centers (out of 6 C). D-glucose is one of the 16 stereoisomers (2^n, where n represents the number of stereo-centers). Intramolecular reactions between aldehyde and hydroxyl groups form a cyclic hemiacetal, in which a new stereo-center is created (the anomeric carbon C1). Depending on the position of the hydroxyl group on the C1 when compared to the terminal CH_2OH, D-glucose can have either α (opposite side) or β (same side) configuration. As a repeating unit, connected via α or β glycosidic linkages, D-glucose appears in the form of the four most abundant and most consumed polysaccharides on earth, amylose, cellulose, amylopectin or glycogen. [1] Amylose is a linear, water insoluble polymer usually constituting 20% to 30% of starch depending on its origin, in which glucose units are linked via α(1→4) linkages. [2] It plays a role in plant energy storage and the degree of polymerization in different plants can vary between few hundreds and several thousands. [3] Amylose can take three main forms, disordered amorphous and two helical conformations (single and double-stranded helix). The helical conformation facilitates amylose to be a host for small guest molecules and allowing it to, for instance, slowly release specific substances with time, which can be used among other applications in medical purposes. [4, 5] Additionally it is used in both industrial and food-based processes where thickeners, gelling agents or water binders are needed. Amylopectin is a branched, water soluble α(1→4) glycosidic polymer found in starch, next to amylose. Branching takes place at α(1→6)

points. With a degree of polymerization between 60,000 and 6,000,000, it belongs to the longest polymers found in nature. [6] Amylopectin has long branches of up to 30 glucose units, clustered branching points, and is widely used in food and cosmetic industry. [7, 8] Glycogen is “animal starch”, with much shorter branches up to 11 glucose units, and random branching twice as high than in amylopectin. [9] Cellulose is one of the constructing units of primary cell walls in green plants. It is the most abundant polymer in nature. [10] Its degree of polymerization varies from few hundreds up to 10 000. [11] Cellulose is mostly produced in nature as crystalline cellulose in which glucan chains are parallel to each other, and is described as cellulose I. Some organisms can produce cellulose in which glucan chains are oriented antiparallel, so called cellulose II. [1] Cellulose is mainly used in paper industry.

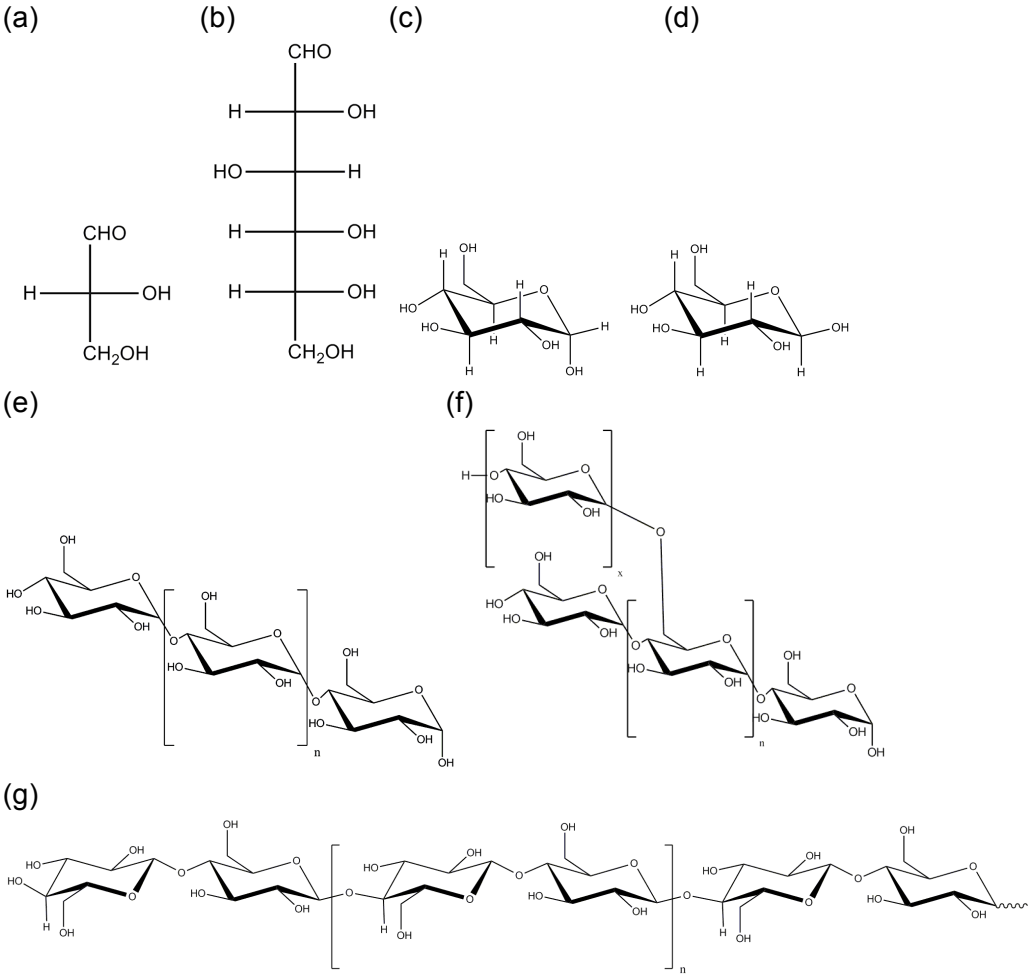

Figure 1.1. Chemical structures of **(a)** D-glyceraldehyde, **(b)** D-glucose, **(c)** α-D-glucose, **(d)** β-D-glucose, **(e)** amylose, **(f)** amylopectin and **(g)** cellulose.

Due to polysaccharides excessive existence in nature and use by human beings it is very important to understand the link between the structure of the polysaccharide and its behavior. Additionally their meticulous characterization in respect to the molar mass, various distributions, structure, the type and the degree of branching (if applicable) is of great importance. Regardless of their simplicity, they are very hard to characterize. For instance, branched polysaccharides such as amylopectin differ to a large extent from their linear analogues (amylose).

Over the years the synthesis of well-defined molecular architectures, such as different dendrimers, [12-14] or star branched polymers, [15] and their characterization contributed to the understanding of their structure/property relationship. Following this reasoning, the first topic in this chapter focuses on the synthesis of well-defined oligo- and polysaccharides. Multiple repetition of a specific stereo- and regio-selective glycosylation makes the synthesis of polysaccharides very complex using conventional organic chemistry. However, in the last few decades a new pathway of synthesizing structurally well-defined polysaccharides was established; using enzymes *in vitro* as catalyst.
Complicated structures such as branched polysaccharides show differences in solubility, rheological and mechanical properties when compared to their linear analogs. In order to establish improved protocols for the characterization of these polysaccharides, which is the second topic in this chapter, well-defined standards are required.

1.2 Easier and greener! Enzymes as catalysts in the synthesis of polysaccharides

In living systems, enzymes play the role of catalysts and are responsible for the majority of biosynthetic processes. Nearly all, natural macromolecules are synthesized under mild conditions with respect to temperature, pH, pressure, etc. These reactions achieve high conversion rates and almost absolute regio-, enantio-, chemo-, and stereoselectivity, resulting in little or no side products. [16] If these characteristics are transferred to *in vitro* enzymatic polymerizations, outstanding control of the structure and the creation of novel structures with biodegradable properties in a clean, selective process using less energy is expected. Therefore, regardless of being a big mystery in many chemistry-related fields for already many decades, enzymatic catalysis has been widely explored in the sense of finding new enzymes or unraveling the mechanisms of the known ones. [17]

The Enzyme Commission acknowledges 6 main groups of enzymes (oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases) out of which the first three are currently utilized as catalysts in enzymatic polymerizations. Enzymes from the group of hydrolases, mainly glycosidases have been extensively utilized for the synthesis of polysaccharides. [18, 19] Glycosidases are involved in both reactions of the hydrolysis and the reverse reaction of bond-formation, unfortunately only with glycosyl fluorides, which are very expensive and can be toxic. [18, 20] Therefore, for this work, the most important enzymes are from the transferase group, since creation of a glycosidic linkage in the biosynthesis of polysaccharides is mostly catalyzed by glycosyltransferases employing the corresponding sugars (monomers and primer if needed). [19]

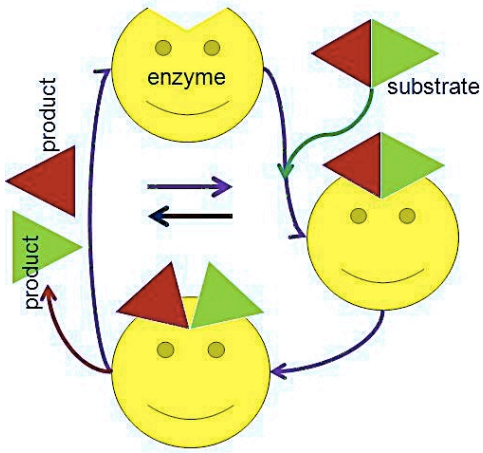

Figure 1.2. Enzyme-substrate relationship: the “key and lock” theory

One of the two fundamental features of enzymatic catalysts for their use in polymer chemistry is the “key and lock” relation between enzymes and substrates used in biocatalysis (Figure 1.2.). [21] The product is created by the accelerated bond formation of the activated substrate, which is previously locked in the enzyme. [19] The second important feature provides an explanation for the reaction progress at mild temperatures and concerns the activation energy. Due to the stabilized transition state the activation energy of enzymatic polymerizations is much lower than in non-enzymatic one, as depicted in Figure 1.3. [22] The rate acceleration of enzyme-catalyzed reaction ranges between 10^6 to 10^{12}, nonetheless in some specific cases can be as high as 10^{20}. [23]

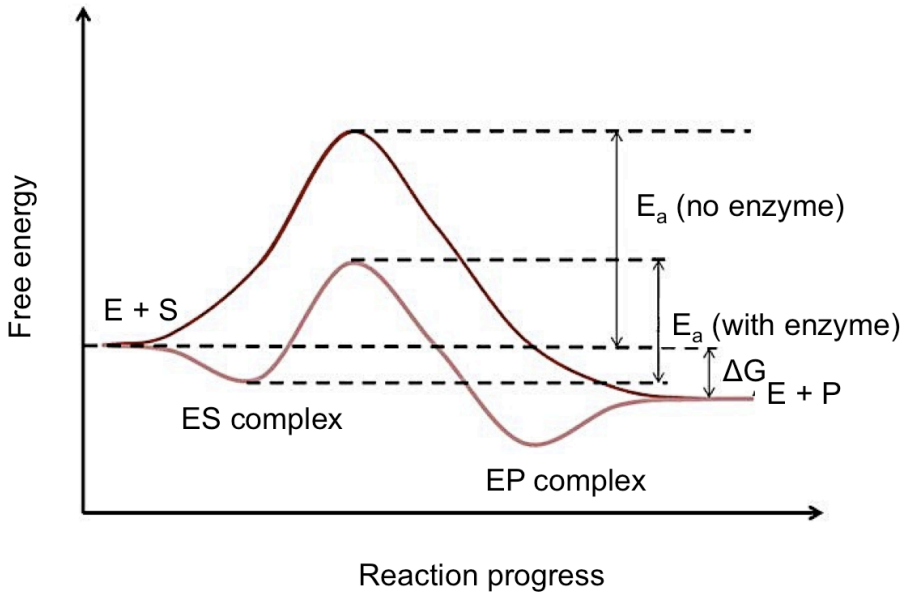

Figure 1.3. Transition state diagram (E enzyme, S substrate, P product, ES is enzyme-substrate intermediate, EP is enzyme-product intermediate, E_a is the energy of activation)

1.2.1 Amylose

In nature, linear glucans such as amylose are synthesized from ADP-glucose by the group of enzymes called synthase (EC 2.4.1.1). Unfortunately both enzymes and the monomers are very unstable. [24, 25] Therefore, amylose is usually synthesized *in vitro* with certain types of active phosphorylases (EC 2.4.1.1) that belong to the group of transferases. [26, 27] In nature, phosphorylase catalyzes phosphorolysis at the non-reducing end of the glycosidic linkage; however it can be used *in vitro* to catalyze glycosylation reactions and the formation of glycosidic linkages due to relatively low bond energy of a glycosyl-phosphate. [28] Pure, linear amylose can be synthesized under appropriate conditions by the phosphorylase-catalyzed polymerization of glucose-1-phosphate with the release of inorganic phosphate. These reactions are initiated from a few glucose units long primer, depending on the phosphorylase source. For example, in the case of potato phosphorylase a primer of at least three glucose units is needed to start the reaction. [29]

Pfannemüller et al. synthesized various amylose derivatives via enzymatic polymerization using potato phosphorylase and modified primers, and opened up a new pathway in the synthesis of amylose-based polymers. [30, 31] The use of modified primers in this synthesis offered an opportunity for the synthesis of amylose-based polymers, which are of potential interest for use in different fields of industry. Phosphorylase was able to start polymerization from synthetic substrates, such as ω-methoxy poly(ethylene oxide)-primer, [32] maltopentaose-substituted styrene, [33] or maltoheptaose coupled to butane diamine (BDA), tris(2-aminoethyl)amine (TREN) and amine functionalized polyethylene glycol (PEG) and create interesting glycoconjugates. [34] The enzymatic polymerization of amylose-block-polystyrene was also possible and started from maltoheptaose-modified polystyrene. [35] Even grafted polymers were synthesized using the same reaction, when well-defined maltoheptaose-grafted chitosan was used as a primer, [36] or materials such as amylose-grafted poly(dimethylsiloxanes). [37] Additionally, many complexes between amylose and guest molecules could be obtained very elegantly *in situ* during the enzymatic synthesis of amylose catalyzed by phosphorylase via so called "vine–twinning polymerization". [38, 39] This method was used to create complexes such as amylose-poly(ε-caprolactone) complexes, [40, 41] or amylose-polytetrahydrofurans (PTHF) in the case of low molar mass amylose. [42] Moreover, this synthesis was successful in the presence of various synthetic polymers such as polyethers, polyesters, poly(ester-ether), and amphiphilic block copolymer by using diethyl ether as a two-phase system. [43] An important factor for the creation of inclusion complexes is the hydrophobicity of the guest polymers. Hydrophilic polymers, such as polyethylene glycol, are not able to form a complex. [39] When "vine- twinning polymerization" was performed in a mixture of PTHF and poly(oxetane), due to the higher hydrophobicity the synthesized amylose favorably formed a PTHF inclusion complex. [44] Complexes created when a different primer and a substrate were used for the phosphorylase-catalyzed reaction, maltooctaose and maltooctaose-phosphate, consisted of a polymer with the length regulated by the length of a guest polymer. [45] During the last year, two different interesting methods for amylose-inclusion complexes were established for PTHF and styrene. The amylose−PTHF complexes were created by direct mixing using water as a medium. [46] Whereas the formation of amylose−polystyrene inclusion complexes were created by a two-step

procedure where the styrene is in the initial step inserted into amylose, and polymerized via free radical polymerization inside amylose in the second step. [47]

Transferases are not the only family used for the enzymatic synthesis of amylose. Glycosidases, for example α-amylase (EC 3.2.1.1) from the hydrolase family were also successfully used for the oligo-amylose synthesis. In nature, glycosidases in the presence of water catalyze the hydrolysis of glucans. Since these reactions are reversible, under appropriate conditions, with suitable substrate they can catalyze the formation of glycosidic bonds. α-amylase can be used as a catalyst in amylose synthesis from α-D-maltosyl fluoride in a mixed solution of methanol and phosphate buffer. [48] Amylosucrase (EC 2.4.1.4), from the glycosyltransferase family catalyzes the transfer of glucosyl units to an acceptor molecule without any primer. [49] When amylosucrase from *Neisseria polysaccharea* was used as a catalyst in amylose synthesis, the degree of polymerization up to 58 glucose units was achieved. [50]

When a primer was used, for example glycogen, and a high initial sucrose/glycogen ratio, dendritic nanoparticles were created. The external glycogen chains were enzymatically extended, and simultaneously the synthesis of linear amylose occurred and resulted in semi-crystalline fibrous entities. [51]

Branched or hyper-branched amylose

Amylopectin and glycogen are essentially amylose that is branched. Depending on the used enzymes the degree of branching can vary. Highly branched amylose could be synthesized using potato phosphorylase and *Deinococcus geothermalis* glycogen branching enzyme (Dg GBE, EC 2.4.1.18) via an *in vitro* tandem polymerization from glucose-1-phosphate (G-1-P) as the substrate and maltoheptaose as the primer. [52] Dg GBE is known to branch amylose with an atypical side-chain distribution to a high degree of branching of 11%. [53] When phosphorylase *b* is used instead of the potato phosphorylase, Dg GBE is known to branch amylose even higher, up to 13%. [54] It was shown that the branched amylose with substantial degree of branching (2.1 to 4.5%) could be synthesized with maize starch-branching enzyme IIa in a two-step reaction. The degree of branching depended on whether the short chain amylose or the long the chain amylose was used as a substrate for the branching enzyme. [55]

When different substrates such as sucrose were used, with maltotetraose as a primer, three enzymes were needed for the successful synthesis: sucrose phosphorylase (EC 2.4.1.7), phosphorylase and a branching enzyme in this case from *Aquifex aeolicus*. [56] It was also shown that synthetic glycogens, which closely resemble native glycogens, could be synthesized from short-chain amylose using branching enzyme and amylomaltase (EC 2.4.1.25). [57] Other ways to produce synthetic glycogens were obtained either in tandem by using Neisseria polysaccharea amylosucrase to produce linear chains and Rhodothermus obamensis branching enzyme; [58] or by using the Rhodothermus obamensis branching enzyme alone and different kind of primers, linear or branched ones. [59]

1.2.2 Cellulose

Synthesizing cellulose *in vitro* has been challenging for many decades, especially for traditional organic chemistry, due to frequently needed repetition of glycosylation reactions in a regio- and stereoselective way. With a lot of effort, organic synthesis was successfully performed by cationic ring-opening polymerization of 3,6-di-O-benzyl-α-d-glucose-1,2,4-orthopivalate, with a degree of polymerization of around 20. [60, 61] Nevertheless, the first *in vitro* enzymatic synthesis was performed more than 20 years ago. This polymerization occurred in a mixed solvent formed of acetonitrile and acetate buffer; and was catalyzed by cellulase (EC 3.2.1.4) from *Trichoderma viride* while β-D-cellobiosyl fluoride used as substrate. [62] In nature, cellulase can catalyze the hydrolysis of β(1→4) glycosidic linkages, however as previously mentioned for the enzymatic polymerizations of α−glucans, if used under appropriate conditions cellulase can catalyze the reverse reaction. Cellulase is substrate-specific, and many authors used this property to synthesize oligocellulose from sugar fluorides without protecting groups. [63, 64] The synthesis procedure of longer-chain cellulose from cellobiose with a cellulase/surfactant (dioleyl-N-d-glucona-l-glutamate) complex in a non-aqueous solvent (lithium chloride in dimethylacetamide) was successfully performed due to functional preservation of cellulose in the complex. A degree of polymerization of over 100 was achieved, regardless of the extremely severe conditions for the enzyme. [65]

Another choice to synthesize oligocellulose is by using sugar phosphorylases, since some of these enzymes also catalyze the formation of β linkages. Both labeled and non-labeled cellobiose was enzymatically synthesized for the first time with cellobiose phosphorylase (EC 2.4.1.20) almost two decades ago. [66] Unfortunately, no work with cellobiose phosphorylase showed oligocelluloses bigger than 2 glucose units when glucose is used as a primer. Cellodextrin phosphorylase (EC 2.4.1.49) has also been found in *Clostridia* and was reported to catalyze the formation of cello oligosaccharides with an average degree of polymerization (DP) of 9 from G-1-P. The primers used in this study were both cellobiose and glucose, and both appeared to be good acceptors for the enzyme. [67] Yields were improved by using a different substrate, glucose-1-fluoride instead of G-1-P. Using various primers with different

glycosidic linkages, resulted in new interesting structures such as combination of β(1→4) and(1→2) or β(1→4) and (1→3) linkages. [68]
Cellulose is the β isomer of amylose, and very hard to dissolve in most of organic solvent, due to strong intra- and intermolecular hydrogen bonding. In the last ten years, the trend of using room temperature ionic liquids (ILs) as solvents is growing, especially in the field of biocatalysis. [69, 70] This relatively new group of solvents has a large potential to be used in green chemistry. They are completely composed of ions, liquid at room temperature, nontoxic and have extremely low vapor pressure. ILs are reported to be less deleterious than organic solvents for the wide range of enzymes. [71] This feature of ILs can be exploited for the enzymatic synthesis of polar and hardly soluble polymers such as cellulose. Previous studies have shown that imidazolium based ILs can efficiently dissolve cellulose. [72, 73] Not only enzymatically catalyzed reactions showed higher enzyme stability, but they have also shown faster rate and greater selectivity. However, they are not super-solvents; they have drawbacks for the synthesis such as very high viscosity or stringent control on water activity and pH. [74] Recent studies showed that hydrolysis of cellulose catalyzed by cellulases could occur successfully in 1-methylimidazolium chloride (MIM-Cl) and tris-(2-hydroxyethyl)-methylammonium methylsulfate (HEMA). [75] These results open new ways in the future of enzymatic synthesis of cellulose.

1.3 Oxymoron: simply hard to characterize!

1.3.1. Starch (amylose and amylopectin) and glycogen

Starch, glycogen and their derivatives, have an intricate structure, which includes four basic structural levels: from single molecules to large supra-molecular complexes. [76] The first level gives information concerning the nature of the monomers, which compose the polysaccharide. The second level corresponds to the substitution or the branching pattern of the polysaccharide. Whereas the third level structure provides data on different architectures that can occur in the whole polysaccharide macromolecule. In this level macromolecules with various structures from either simple, linear cellulose molecules, slightly branched amylose molecules; via grafted saccharides such as derivatives of cellulose and starch or linear polymers with alternating bonds such as β-glucans to the most interesting ones - hyper-branched amylopectin molecules or glycogen molecules can be found. The fourth level tells us more about supramolecular organizations of the molecules with architectures such as starch (amylose and amylopectin) and their interaction with the surrounding.

Numerous techniques can be used to investigate the second level of structure related to either branching pattern or the substitution of the polysaccharide. The degree of branching can be determined by well-established destructive techniques such as a methylation or per-iodate oxidation technique, where the non-reducing terminal glucose units of the external branches produce 2,3,4,6-tetramethylglucose in the methylation method and formic acid in the periodate oxidation procedure. Regardless of the structure, the number of such end-groups is related to the number of branching points like n to n-1. [77] Additionally, enzymatic destructive methods can be performed. In these methods, the glucose units connected via α-(1→6) linkage are uniquely determined. For instance, they can be removed as free glucose by amylo-1,6-glucosidase, and other glucose units are split off such as G-1-P by phosphorylase. Nondestructive techniques such as NMR spectroscopy can provide data on the average degree of branching and the degree of substitution. [78, 79]

The most interesting structures are the ones from the third level for establishment of the characterization protocols for branched polysaccharides. Knowing how complex branched polysaccharides are

with their multi-dimensional distribution of molecules, the structural characterization at this level is an extremely challenging task. [76] Size separation techniques coupled with different detectors could be one of the best choices for solving this perplexing task. They can provide information on the size, mass or number distributions. These distributions tell us more about the individual structural characteristics of molecules.

Currently, the most developed method and definitely the most popular method for determination of previously mentioned distributions of such polymers is size-exclusion chromatography (SEC) often called gel-permeation chromatography coupled with multi-detection. [80] The separation of macromolecules via SEC is solely based on the hydrodynamic volume (V_h) of the characterized molecules. [81] In the SEC analysis, the dissolved analytes are injected into a continual flow of solvent - mobile phase. The mobile phase flows through a stationary phase which consists of millions of highly porous, rigid particles, which are tightly packed together in a column. The pore sizes of these particles are controlled and available in different size ranges and materials.

For SEC separation, the hydrodynamic volume is defined as:

$$V_h = \frac{2}{5}\frac{\bar{M}_n[\bar{\eta}]_w}{N_A} \qquad 1.1$$

where $\bar{M}_n$, $[\bar{\eta}]_w$ and N_A are respectively the number-average molar mass, weight-average intrinsic viscosity of a polymer and Avogadro's number. [76] In an ideal case, the size separation is based exclusively on size and no interaction between molecules and the column material should exist. When the size-separation regime is established, larger molecules will elute first since the smaller ones will stay longer between and inside the column pores. Typical solvent systems for polysaccharides are polar aprotic solvents, such as DMSO or dimethyl acetamide, with an addition of hydrogen bonding disrupting salts (LiBr/Cl) to minimize the interaction with the column, [82, 83] water-based ones, [84] or even their mixtures. [85] The range of SEC separation is very wide, dependent on the pore exclusion limit of the columns offered by different manufacturers. The larger the molecules are, the more critical the interpretation of the data is due to shear scission. [86] Nevertheless, larger molecules can still be characterized with some limitations. [82]

It is established for a range of branched polymers that the calibration curve in terms of V_h (see Equation 1.1), determined with a set of linear

polymer standards is equally valid for branched polymers (so called principal of “universal calibration”). [81, 87-89]
Therefore the universal size calibration is mostly performed using a series of linear polysaccharide standards (e.g. pullulan) with narrow dispersity and known molar mass to results in the relation between elution volume (V_e) and V_h. The V_h for a linear polymer used as a calibration standard can be determined experimentally from its $\overline{M}_n$ and $[\overline{\eta}]_w$, when coupled viscometer and concentration detector are used. However if the viscometer is not available or applicable for various reasons, the calibration can still be performed when the Mark–Houwink parameters K and α are known for the standards in the analytical solvent at the separation temperature. The calibration range is usually covered with standards up to R_h of 50 nm; therefore extrapolation is needed for larger molecules. Even though it is done frequently, one should take care of the accuracy of these results. This problem can be reduced for larger molecules when light scattering measurements are performed; the relationship between elution volume and the radius of gyration (R_g) is established and used as complementary information for the universal calibration. Nonetheless, the relationship between the two radii used for different calibrations (R_h and R_g) depends on the shape and the branching structure of branched polymers and is not well-investigated and established for hyper-branched polymers such as glycogen or amylopectin. [90] The most accurate solution would be the combination of calibrations, one sensitive and accurate for smaller molecules and one for larger ones. For instance MALLS with on-line QELS is a very good combination (see Chapter 6). Whatever calibration is used, it should be emphasized that analysis of extremely large molecules with SEC is critical and questionable due to shear scission, poor separation and band-broadening issues. [76] Even though column development is ongoing, a column or an optimum combination of columns that will provide good separation of amylose and amylopectin and their correct distributions (separately in one run) still does not exist. However, when enzymatically synthesized amylopectin analogs were used, with a R_h below the size SEC-limitations, not only a good characterization of different distributions was achieved but parts of the enzymatic mechanism were solved with the assistance of matrix-assisted laser desorption ionization-time-of-flight

mass spectrometry (MALDI-ToF MS), which is further explained in detail in Chapter 4. [84]

As an assisting technique to SEC packed column hydrodynamic chromatography (HDC) could be used. HDC is in a way SEC without pores in the packaging beads, and therefore the separation mechanism is caused by the parabolic velocity profile, in which small particles lie near the wall (low-velocity flow regions) and the center of mass of the large ones stay closer to the center of the tube where the fastest flow is. [91, 92] The efficiency of the separation is dependent on the ratio between the macromolecular size and the channel size. When coupled to the same detectors like SEC, HDC should in principle result in similar size distributions, with a lower selectivity, when suitable calibration is applied. Some analysis of starch using HDC in a mixed-bed particle column and temperature gradients in aqueous mobile phases have been reported, unfortunately full separation between amylose and amylopectin was not attained with mono-sized columns. [93] However, if the column was packed with a mixture of 5 and 19 μm glass beads and the column temperature was decreased to 3 °C, results comparable to SEC analysis were attained.

It is known that HDC effects may occur for the largest polymers in the SEC calibration range if pore diameters of the SEC packaging are relatively large in comparison to the particle diameter. Therefore a combined HDC-SEC measurements should be used, in which the smallest molecules are separated based on a SEC mechanism, and the largest molecules show differing migration rates due to HDC effects. [94] Recently the measurements of various starches were performed successfully by using high pressure SEC (HPSEC) columns with very small pore sizes (no penetration of the macromolecules into the pores), which resulted in the combined effect of HDC in the void volume and SEC later on. However, when compared to asymmetric field-flow fractionation (AF4) analysis, which will later on be explained in more detail, the results matched but AF4 enabled a better separation of amylopectin (Figure 1.4.). [95] Nevertheless, the use of HDC for characterization of branched polysaccharides should be investigated more to overcome the drawbacks in SEC analysis, especially the fact that amylopectin is submitted to shear scission in SEC stationary phase which leads to an underestimation of amylopectin size and that, in combined HDC-SEC analysis, it is eluted at the void volume of the SEC column which can lead to a lower selectivity.

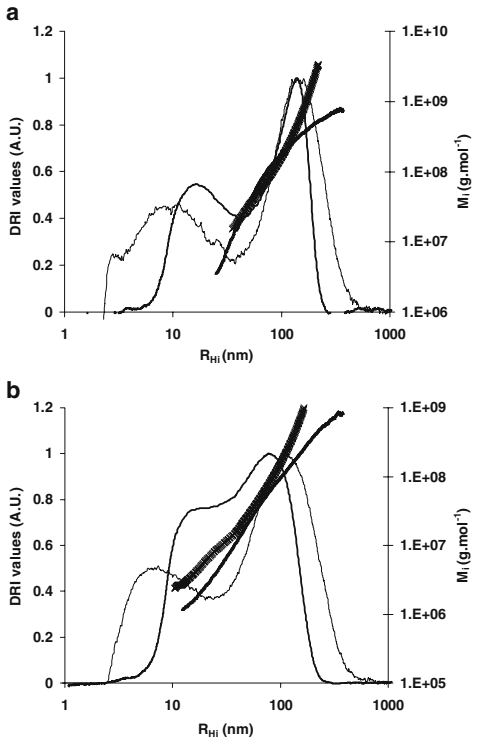

Figure 1.4. Hydrodynamic radius (R_{Hi}) distributions (differential refractive index (DRI) values) obtained using AF4 and HDC-SEC using a Shodex KW802.5 column. **(a)** Wild-type wheat starch (WTWS); **(b)** wild-type rice starch (WTRS). The thin lines represent DRI values obtained using AF4, the thick lines indicate DRI values obtained using HDC-SEC. The small triangles and large crosses represent the molar masses (Mi) obtained using AF4 and HDC-SEC, respectively.
Reproduced with permission. [95] Copyright 2011, Analytical and Bioanalytical Chemistry

For the separation and characterization above the size limits of SEC, field-flow fractionation (FFF) is another technique that has been applied. It is an elution based chromatography technique in which the fractionation occurs in a single liquid phase. The selectivity is based solely on the diffusion coefficient. FFF has no stationary phase and, therefore is seen as an almost universal characterization technique. [96] FFF is categorized by the use of an external field, which is applied vertically to the direction of

the sample elution flow through an empty, thin ribbon-like channel. Due to the high aspect ratio of the FFF channel a laminar parabolic flow profile is developed, in which the separation is caused by the external field. Separation occurs because different molecules move in different flow velocity zones, and can be performed by different mechanisms arising from different opposing forces. Three most commonly used modes in all FFF techniques are normal, steric, and hyper-layer mode; out of which the most used one is the normal mode of separation. [97] The normal mode is based on Brownian motion of the molecules and the diffusion plays an important role in this mechanism (when molecules are smaller than 1 μm) by controlling the distribution across the channel. Smaller molecules accumulate in regions of faster streams and elute before larger molecules. The steric mode exists when the diffusion is negligible (when molecules are larger than 1 μm) and the distance of closest approach to the accumulation wall directs retention. The center of mass of the smaller particles is in the slower flow line (elute later), due to better approachability to the wall. When high flow velocities are used, the hyper-layer mode occurs, because lift forces move particles towards higher velocity streams moving more than one particle radius from the accumulation wall and eluting particles faster than in the steric mode but with the same elution order. [98]

Depending on the external force field, which is applied to create the separation, different FFF techniques can be recognized, among which the most popular are flow, thermal and sedimentation one. Flow FFF (symmetric FFF and AF4) are the most universal of all FFF techniques. The fractionation range covers a broad band of sizes from 1 nm up to 50 μm in both water and organic solvents, depending on the membrane cutoff, the channel height and the separation mode. Flow FFF is used for the analysis of polymers, colloids and particles in that size range. [99] In this case separation takes place in a laminar flow of a membrane, which is caused by a flow perpendicular to the sample elution flow. AF4 is an evolution of symmetrical flow FFF that only uses one permeable wall (instead of two) as the accumulation wall (a frit covered by an ultrafiltration membrane). The structure of glycogen, degraded and modified starch were characterized with AF4. [99-101] AF4 coupled with multi-angle laser light scattering (MALLS) enabled the characterization of amylopectins' structure, with its branching characteristics. This analysis successfully highlighted the differences between amylopectins as a function of

botanical source and was less time-consuming than the other methods generally used. [99] The same technique was very useful for observation of the changes in the size and the molar mass of starch molecules during carboxymethylation (see Figure 4). [100]

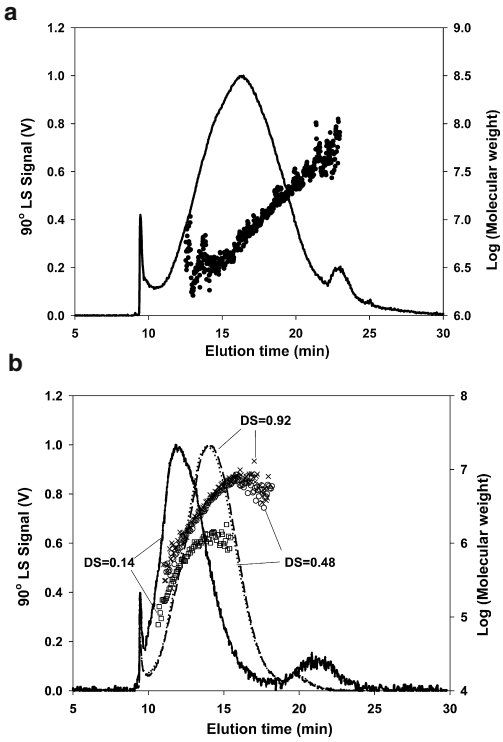

Figure 1.5. (a) AF4/MALLS fractogram of native cornstarch and **(b)** AF4/MALLS fractograms of carboxymethyl starches (CMS).
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AF4 coupled with a concentration detector and MALLS has potential advantages over SEC and other size-separation techniques with stationary phases when native starches are analyzed. For example, degradation is almost completely avoided because the problem of shear scission is suppressed, that does exist in SEC analysis caused by the stationary phase. In AF4 analysis the separation is based on the differences in the diffusion coefficients of the analyzed components. This implies that the separation depends on the size and shape of particles

and molecules. For macromolecules with diameter lower than 1 μm the normal mode is preferred. Nevertheless, when working with large macromolecules it is important to check the fractionation mode that occurs in order to avoid a mixed fractionation (normal and steric/hyperlayer modes). In the case of a mixed fractionation, poor fractionation is achieved due to an inversion of the elution order. Therefore, when using AF4 for size determination (especially if the FFF theory is used), it is important to work in the normal mode, otherwise the fractionation is poor. Additionally, the channel thickness is significant for the working mode. For instance when amylopectins were analyzed with an AF4 with a small channel (thickness 130 *μm)* the hyperlayer fractionation occurred, hence the hydrodynamic radius was much smaller than expected. [102] If the thickness of the channel is satisfying, the fractionation is performed properly, in the normal mode. [99] The limiting obstacle for starches can be the solvent; amylose is water insoluble, and amylopectin molecules aggregate easily in water. AF4 membranes can be sensitive towards harsh organic solvents, or salt containing solvents. New materials are created every day, and it is just a matter of time when membranes with improved solvent resistance are going to be introduced for AF4. Thermal FFF uses a thermal gradient for separation and is mostly used for the analysis of lipophilic shear sensitive synthetic polymers or aggregates in both water and organic solvents. [103] It was also shown to be applicable for the separation of some starches in DMSO, yet difficult for good characterization of branched polysaccharides. [104] Sedimentation FFF in which centrifugal forces are applied is used for particles bigger than 30 nm and very useful for biological applications in both water and organic solvents. [105] With this type of FFF it was possible to determine various distributions of different starch molecules. [106] Additionally, some work showed that it is a very good tool to monitor amylolysis of native starch; and a simple method to monitor biophysical modifications of starch granules. [107]

Size calibration can be established using the theory developed for all types of FFF technology, by relating the retention parameter to the corresponding diffusion coefficient. With known diffusion coefficient, one can use the Stokes–Einstein relation to calculate the hydrodynamic radius. [108 109] When choosing this type of calibration it should be taken into account that many approximations are made, and large deviations can appear for complex molecules. Other calibrations are also possible,

such as using monodisperse standards, or the hydrodynamic radius from quasi-elastic light scattering (QELS) as a size scale along the elution profile. [95] Most used detectors with FFF are concentration detectors and MALLS, as viscometer detectors are very sensitive towards pressure changes, and unfortunately there are many changes in pressure during FFF analyses caused by different flow profiles. [110]

One of the promising techniques that could provide a completely new structural characterization of branched polymers is molecular-topology fractionation (MTF). This is a relatively new type of chromatographic dilute solution polymer separation, in which the fractionation is based on both topology and molar mass. This type of fractionation can in some cases lead to a co-elution of branched molecules with linear molecules of lower molar masses. Nevertheless, the topological separation of macromolecules is possible above the critical R_h (depending on the morphology of the stationary phase). Fractionation occurs by elution in narrow channels, which have the dimensions similar in size to the dimensions of the analytes at low flow rates with an applied flow field as the driving force. There are two suggestions for the separational mechanism. In the first one, the rate of transport is detemined by the relaxation modes for reorientation of the molecules, which are restricted by the channels. Whereas in the second the rate of transport is determined by the entanglement of the molecules on the stationary phase. In both cases the largest molecules stay longer in the column, and produce an extra retardation for branched molecules, which leads to a separation by topology, branching and results in branching distribution even for the components with the same R_h. [111]

However, there has not yet been any published research on polysaccharides such as starch, even though it would be a solution for the full separation of amylose and amylopectin or the modified starches depending on their substitution pattern.

Multidimensional chromatographic analyses such as LCxSEC have been successfully performed for the separation of complex synthetic copolymers based on their chemical structure and size. Sadly, solvent gradient LCxSEC are not yet used for the separation of branched polysaccharides such as different starches, due to the lack of differences in their chemical composition. [112, 113] Nevertheless, this problem could be solved with a different combination. Imagine, how perfect a MTFxSEC would be? An equivalent system was used for the separations of star

branched polystyrenes and of randomly long-chain-branched polystyrenes. [114] Unfortunately it was still not used for polysaccharides.
Until a perfect multidimensional separation is established for branched polysaccharides, destructive analysis can be performed, starting with (partial) hydrolysis of the polysaccharide, which is followed by different examination of the products. In this way, information on the constitution of the polysaccharide molecules can be unraveled. The best way to perform the partial hydrolysis is enzymatically, since enzymes cleave specific linkages. [78] For example, isoamylase cleaves specifically α(1→6) glycosidic linkages, therefore can debranch amylopectin or glycogen. After the desired partial hydrolysis, analysis can be performed by mass spectrometric techniques such as MALDI-ToF MS, [84] or different chromatographic techniques such as SEC or high-performance anion-exchange chromatography (HPAEC). [53] More perspective, but also more challenging is multidimensional off-line chromatographic separation. Very interesting work has been published on natural starches with an offline SECxSEC with a debranching step in between. [110, 115, 116] This interesting approach can be applied to any branched polymers that can be selectively debranched.

1.3.2 Cellulose

The natural form of cellulose in cell walls is thin microfibrils, which contain cellulose I nanocrystals (the most common crystalline form of cellulose in nature). The thermodynamically stable allomorph is cellulose II, which has an additional hydrogen bond per glucose residue, and antiparallel chains, but is very rare in nature. Microfibrils come in many shapes and sizes, and create the cell walls. The detailed structure of the cell wall can be studied by numerous techniques. One of the techniques is synchrotron scanning X-ray microdiffraction, which can result in a picture of the arrangement of the fibrils. [117, 118] If higher resolution is needed, a combination of atomic force microscopy (AFM) and scanning electron microscopy (SEM) can be successfully used. [119]
Concerning cellulose characterization, an additional problem appears if one has a need to characterize noncrystalline or amorphous domains. For example, when wide-angle X-ray diffraction (WAXD) is used for determining the crystalline forms and the crystallinity, in most cases WAXD provides a diffuse diffraction pattern, which is considered as

amorphous cellulose. [120] Some researches tried to determine the non-crystalline regions using FTIR monitoring of the deuterated hydroxyl groups, [121] and succeeded when FTIR monitoring of the deuterated hydroxyl groups led to the two-dimensional correlation spectroscopy analysis. [122] Additionally, solid-state cross-polarization magic angle spinning carbon-13 nuclear magnetic resonance (CP/MAS ^{13}C-NMR) can be used to provide information on the type of hydroxymethyl conformation at the C6 position. [123] The type of hydroxymethyl conformation at C6 presumably determines the extent of crystallization, as well as the definitive morphology of cellulose. [124] The conformation of C5-C6 and their interactions can differ. When the rotational position of hydroxymethyl groups at C6 is considered as non-oriented, one can assume to posses the non-crystalline region of cellulose; whereas when they are all identical one is sure to have the crystallites. [125]

The fact that cellulose usually has a highly ordered crystalline structure prevents solvents to easily penetrate into the fibers and to disrupt the intermolecular hydrogen bonds. This makes cellulose one of the most challenging materials for characterization. The majority of solvents for cellulose are multi-componential, out of which many are very aggressive towards most equipment parts. The average degree of polymerization of low molar mass cellulose was easily determined via ^{1}H-NMR spectroscopic analysis up to a Dp of 15 (oligocellulose was dissolved in 4% (w/w) NaOD–D_2O). [67, 126] In case a specific percentage of NaOH can solubilize synthetic or degraded cellulose, distributions and molar mass can be obtained with SEC for instance in water-based solvents. One of the solvents used for natural cellulose in SEC analysis is cadoxen (CdO in aqueous ethylenediamine). Good as a solvent, but very detrimental towards the equipment. [127] In the last 20 years, DMAc/LiCl has been the solvent of choice for cellulose SEC analysis. Nevertheless, many problems and questions arise concerning the reliability of the results. [128]

Alternative solvents were suggested for SEC analysis. Some of these solvents are supercritical fluids and ionic liquids. Supercritical fluids have good solvating properties due to their specific combination of density (liquid); and viscosity and possibility to effuse (gas). [129] Carbondioxide (CO_2) is widely used in polymer science due to its low cost, low toxicity, low critical point and for not being flammable. [130] Some SEC of polystyrene was done with enhanced-fluidity mobile phases, such as THF/liquid CO_2. The addition of liquid CO_2 to THF reduced the viscosity of

the mobile phase. Up to 40% addition of CO_2, increased the chromatographic efficiency; a lower pressure drop across the column occurred; and shorter analysis times were observed. Nevertheless, higher CO_2 proportions resulted in many non-exclusion interactions. [131] CO_2 was also used in a combination with methylene chloride for the SEC study of polystyrenes. [132] One of these combinations, or even a different solvent used commonly for cellulose in combination with CO_2 could enhance the characterization of cellulose. If one considers ionic liquids for the synthesis of cellulose, why would not we use them as liquid phase for SEC separation? An ionic liquid high performance liquid chromatography (HPLC) set-up has recently been produced in Japan and successfully used in cellulose analysis. [133] Unfortunately, no commercially available instruments exist yet, and we do think that manufacturers should see the potential ionic-liquid chromatography, and put more effort into the production of such instruments.

In order to skip solvent problems, cellulose derivatives were used, instead of cellulose, over the last decades, since they are more soluble in organic solvents. However the process of modification can degrade the polymer or change its distribution. Derivatives such as trinitrate or tricarbanilate esters of cellulose were commonly used in the past for SEC analysis, with trinitrate being the first choice. [134]

Our surrounding is made of cellulose, which exactly shows us how good the material is, but as seen from this brief overview of its characterization the more perfect the material is made by nature the more complicated it is for us to understand it completely.

1.4 Aim of the thesis

It is interesting that enzymatically catalyzed reactions and polysaccharides such as starch and cellulose are all around us and still are such a mystery. However, if we combine enzymatic polymerization with characterization, we are on the right track to completely understand these polysaccharides. Additionally, if we compare conventional organic synthesis with enzymatic, not only it is easier and less time consuming to synthesize polysaccharides this way but also many features of enzymatic synthesis are green. For the industrial manufacture the question of costs is still a problem, nevertheless because of the green character enzymatic catalyzes are considered nowadays more and more.

Therefore, the aim of this thesis was to synthesize a variety of highly defined branched polysaccharides by enzymatic polymerization using phosphorylase *b* and Dg GBE as catalysts via a one-pot synthesis. Phosphorylase *b* polymerizes linear amylose and Dg GBE introduces the branching points. Newly introduced branching points, later on serve as new “starters” for the phosphorylase. The tandem polymerization using the two enzymes can be seen in Figure 1.6.

These polysaccharide model systems were used to design improved non-destructive characterization techniques for branched polymers based on for instance the fact that branching reduces molecular size (e.g., at a given molar mass the molecular size decreases with an increasing degree of branching).

In order to achieve this goal and to acquire data that is sensitive to the structure of polymers we used size separation techniques (e.g., size-exclusion chromatography (SEC) and field-flow fractionation (AF4)) with multi detection (e.g., simultaneous differential refractive index measurement, multiple-angle light scattering, and in-line viscometry) in this research.

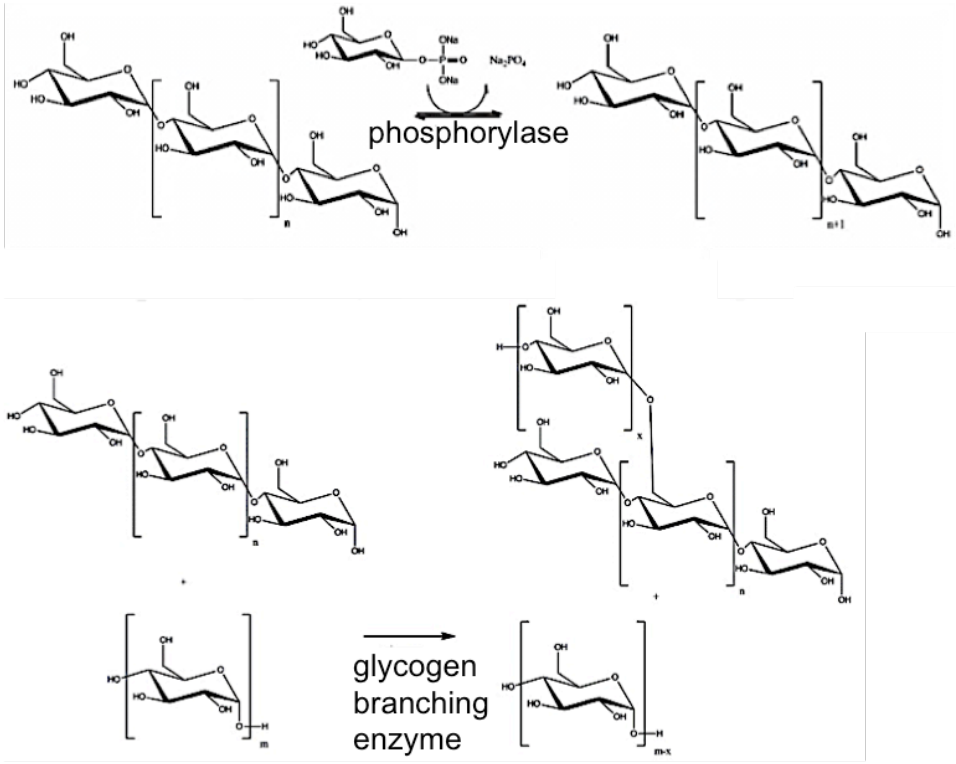

Figure 1.6 Tandem enzymatic polymerization: the actions of phoshorylase and glycogen branching enzyme, respectively.

1.5 Outline of the thesis

This thesis focuses on both the synthesis of branched polysaccharides and their characterization. In Chapter 2 different phosphorylases were estimated for the enzymatic synthesis of amylopectin analogs. The previously established synthesis was modified and the new one was optimized. After establishing the best synthetic pathway using enzymes, a variety of highly defined branched polysaccharides was synthesized in Chapter 3. Several methods for tuning the degree of branching were found and the best one was chosen for further work. Among other techniques, the molar mass of the synthesized polymers was calculated from a quantitative spectroscopic determination of the released inorganic phosphate while the degree of branching of the synthesized polymers was determined by ^{1}H-NMR.

Other chapters focus on characterizations of the synthesized amylopectin analogs. In order to acquire data that is sensitive to the structure of polymers we used size separation techniques ((SEC (Chapter 4) and AF4 (Chapter 6)) with multi detection (differential refractive index measurement, multiple-angle-light scattering; and additionally in-line viscometry in the SEC case and QELS in the AF4 case). In Chapter 4, SEC with multi detection analysis of amylopectin analogs not only provided valuable information concerning different distributions, but also in the combination with the determined polydispersity of the branches using MALDI-ToF we were able to unravel parts of the mechanism of the enzymatic synthesis. The AF4 analysis fully supported conclusions made by SEC, and gave some extra information in regard to the polysaccharides structure, which can be found in Chapter 6.

Additionally, in order to establish as good as possible characterization protocols for the improved techniques, physical properties of synthesized polysaccharides were determined (Chapter 5).

We are convinced that the characterization via this combination of techniques could open up complete new insights into the characterization of branched polymers. Nevertheless, to overcome challenges in characterization in future, it is important to concentrate on finding new solvents such as ionic liquids, which are powerful but not detrimental and on the improvement of promising size separation techniques such as MTF that can provide missing pieces in this large puzzle of molecular structure / physical properties relationship.

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CHAPTER 2

POTATO PHOSPHORYLASE
VS
PHOSPHORYLASE B

The advantages of isolation of potato phosphorylase with protease inhibitor compared to the standard isolation procedure are presented in this chapter. A parallel study of potato phosphorylase and phosphorylase b from rabbit muscle used in the synthesis of glycosidic polysaccharides showed that phosphorylase b shows affinity towards branched substrates. Branched polysaccharides synthesized with phosphorylase b are cleaner and have a higher average degree of branching when compared to branched polysaccharides synthesized with potato phosphorylase.

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2.1 Introduction

Synthesis of branched polysaccharides by the action of potato phosphorylase and *Deinococcus geothermalis* glycogen branching enzyme (Dg GBE) in a tandem reaction was appealingly explained by van der Vlist *et al*. [1] The phosphorylase catalyzed synthesis requires the presence of a recognition unit suitable to start the polymerization ("starters") which is a co-substrate such as starch, glycogen or oligosaccharides. [2-4] Potato phosphorylase polymerizes linear amylose and Dg GBE introduces the branching points. Newly introduced branching points, later on serve as new starter for the phosphorylase, see Figure 1.6. The reaction speed differs substantially with different primer lengths, which results in a broad molar mass distribution. [5] In order to synthesize materials with constant length, using monodisperse primers in the synthesis is very important. One way to synthesize a monodisperse primer is via acid catalyzed hydrolysis of cyclodextrins. In this ring opening reaction a 7-membered cyclic dextrin, β-cyclodextrin is transformed into a linear maltoheptaose (G-7). [6]

The amount of consumed glucose-1-phosphate (G-1-P) can easily be followed by quantitative spectroscopic determination of inorganic phosphate (Pi) since one Pi is released for each consumed G-1-P. This makes the calculation of the degree of polymerization (DP) and molar mass of the synthesized polymer possible at any stage of the reaction. Whereas the properties of branched polysaccharides such as amylopectin (the DP, the degree of branching (DB), the average branch length and the ratio of α/β anomers) can be quantitatively determined from ^{1}H-NMR analysis. [7, 8] Since polysaccharides synthesized in this thesis are amylopectin analogues this method is used as the most suitable one for the determination of the DB.

The first obstacle in the present research was the successful isolation of potato phosphorylase with high activity and the reproducibility of this procedure. The second problem was potato phosphorylase's instability with time, namely not sufficiently long lifetime in suspension, which resulted in need for the frequent potato phosphorylase isolation. Therefore the isolation process needed a slight modification. During the phosphorylase release, proteases are also released. Proteases are enzymes that hydrolyse the peptide linkages in all proteins, including in the phosphorylase. In order to protect phosphorylase from proteases, phenylmethanesulfonylfluoride

(PMSF) proteases inhibitor is added to the potatoes at the beginning of the shredding process. PMSF is a widely used serine protease inhibitor. [9]
Thirdly the polysaccharides were not pure enough for the present research since potato phosphorylase was isolated directly from potatoes and transferred some impurities from the tubers (starch, proteins and other high molar mass compounds) to the products. When dissolved in water, the solution was never completely clear and colorless but opaque and yellowish due to the present residues. Additionally, it seemed not possible to change the DB with this polymerization method.
As standards for improved characterization techniques require the highest possible purity and solubility in the applied solvent, likewise a variation in the DB, the synthesis also required a slight modification. Therefore the second step of modification was to explore possibilities of a different enzyme that will catalyze the synthesis of branched polysaccharides and to characterize the properties of the synthesized polymers. [6]

2.2 Experimental

2.2.1 Materials and methods

α-D-glucose-1-phosphate disodium salt hydrate (G-1-P, Sigma-Aldrich), tris(hydroxylmethyl)amino metane (Tris, Sigma-Aldrich), sodium citrate tribasic hydrate (Sigma-Aldrich), ammoniummolybdate (Sigma), potassium disulfite (Sigma), sodium sulfite (Merck), metol (Fluka), ammonium sulfate (Merck), sodium bisulfite (Acros), p-xylene (Merck), sodium azide (Merck), sodium acetate (Sigma-Aldrich), phenylmethanesulfonyl fluoride (PMSF, Sigma-Aldrich), phosphorylase *b* from rabbit muscle (Sigma-Aldrich), adenosine monophosphate (AMP, Sigma-Aldrich) and dithiothreitol (DTT, Sigma-Aldrich) were used as purchased. Potatoes were obtained from a local store. Dg GBE was kindly provided by R.J. Leemhuis and L. Dijkhuizen.

Spectroscopic phosphate determination

A spectroscopic method for the determination of Pi in blood and urine was established by Fiske and Subbarow. [10] This method is based on formation of a blue phosphate molybdate complex, which can be reduced by a reducing agent forming phosphomolybdic acid and therefore blue molybdous compounds. Concerning the reducing agent, it was found that absorbance produced by p-methyl-amino-phenol sulfate (metol) was significantly more stable than that produced by the amino-naphthol-sulfonic acid. [11, 12] Metol has excellent stability; low absorbance for blanks and absorbance is not interfered by oxalate, citrate, fluoride, etc. Metol was also the reducing agent for color development of choice in the Delsal-Manhouri [13] and Drewers [14] procedure. Therefore the modified method uses metol as a reducing agent for color development and in order to enable molybdate to be reduced on its own and form the complex, very low pH has to be maintained during the procedure. [15] After complexion of all Pi pH is adjusted in order to protect labile phosphate esters such as G-1-P from hydrolysis. [16]

Solution preparation:

1. Metol/sulfite solution was prepared by adding metol solution (200 mg metol in 1 mL H_2O R.O.) to sulfite solution (25 g pyrosulfite and 1 g sodium sulfite in 60 mL H_2O R.O.) and filled with water R.O. to 100 mL. This

solution has to be stored in the dark and the moment it changes the color or starts to precipitate it should be discarded. (reducing agent)
2. Molybdate solution was prepared by dissolving 12.5 g ammonium molybdate in 100 mL H_2O R.O. and adding 125 mL 5 N sulphuric acid while stirring, and filled with water R.O. to 250 mL. (complexing agent).
3. Acetate solution was prepared by dissolving 100 g sodium acetate in 250 mL H_2O R.O. This solution has to be stored in a warm place. (buffer)
Procedure:
10-100 µL aliquot (depending on the concentration of Pi that is expected) was added to a 10mL glass vial. 500 µL solution 1. and 1000 µL solution 2. were added and diluted with H_2O R.O. After 10 minutes 2 mL solution 3. was added. The absorption was measured after dilution to 10 mL total volume after 30 minutes at a wavlength of 716 nm.
UV-Spectroscopy:
UV-Vis measurements were performed with a PYE Unicam SP8-200 spectrophotometer.

^{1}HNMR Spectroscopy

^{1}H-NMR spectra were recorded on a Varian Inova 400MHz spectrometer at 50 °C to separate the HOD signal from the H16 signal properly. 2,2-Dimethyl-2-silapentane-5-sulfonic acid (DSS) was used as an external standard. 10 mg (if possible) samples were dissolved in 700 µL D_2O.
In synthetic amylopectin analogs four types of glucose residues can be observed with protons showing different shifts in the spectrum. Protons from linearly α-(1→4) linked glucose unit with a shift at 5.35 ppm (H14), from α-(1→6) linked unit at 4.94 ppm (H16), from reducing ends at 5.23 ppm (α) and 4.65 ppm (β), and those from a terminal non-reducing end at 5.33 ppm (Ht), see Figure 2.9. The DB represents the comparison of branching points with the number of all linkages, as shown below.

$$\mathrm{DB} = \frac{\int \mathrm{H16}}{\int (\mathrm{H14} + \mathrm{Ht} + \mathrm{H16})} * 100\%$$

Infrared spectroscopy

ATR infrared spectra were recorded on Bruker IFS88 spectrometer with MCT-A as a detector at a resolution of 4 cm^{-1}. An average of 50 scans is presented both for the reference and samples.

2.2.2 Isolation and purification of potato phosphorylase

The process of isolating potato phosphorylase starts by releasing the phosphorylase from potato tubers by shredding pealed and cleaned potato into potato slurry from usually 2 kg of potato with a Universal Machine UMC 5 shredder. Unfortunately for the phosphorylase, proteases, enzymes that hydrolyze the peptidic linkages in proteins, are also released.
Additionally enzymes that catalyse the oxidation of proteins, phenol oxidases are not only found in large concentrations beneath the potato skin but also in the potato itself. [17] Therefore in order to protect the potato slurry from getting black and phosphorylase from being less active sodium bisulfite is added as an anti-oxidant. [18] In both isolation processes, 500 ppm sodium bisulfite and 100 mL citrate buffer (pH 6.2, 50 mM, 0.02% NaN_3) were added to the potato slurry.
The rest of any isolation process was done in an ice bath if not stated otherwise. Each centrifugation step was done at 7500 rpm at 4 °C for 20 min.
After mincing with a UMC 5 shredder, the potato slurry was mixed for 10 min with an ultrathorex blender at 7000 rpm. After pressing this potato slurry through a kitchen towel, a potato juice that consists of a mixture of enzymes was attained. The potato juice is centrifuged in order to remove the remaining solids that went through the towel.
In this mixture of enzymes α-amylase, which can depolymerize amylose, is also present. Hence, removal of α-amylase has to be performed with a heat treatment. At a temperature above 55.5 °C α-amylase denaturates whereas phosphorylase is still stable, which makes the separation of α-amylase from the potato phosphorylase possible simply by 40 min heat treatment at 55.5 °C followed by centrifugation and cooling down.
Isolation of phosphorylase from the juice is done via ammonium sulfate precipitation, which is one of the classical methods for protein fractionation. [19] This specific salting-out technique is based on the principal that enzymes precipitate at different salt concentrations. Firstly, a salt concentration is chosen where all undesirable enzymes precipitate. The juice was mixed with ammonium sulfate (100 g L^{-1}) for 30 min. Secondly the undesired enzymes are precipitated via centrifugation and removed. Thirdly potato phosphorylase was salted-out by adding ammonium sulfate (250 g L^{-1}) to

the supernatant followed by 30 min mixing. Isolation of potato phosphorylase was done via centrifugation. Finally, the isolated potato phosphorylase was re-suspended in citrate buffer (pH 6.2, 50 mM, 0.02% NaN_3).
In order to purify phosphorylase from salt and components smaller than 100 kDa, the suspension was cleaned by the mean of dialysis against citrate buffer (pH 6.2, 50 mM, 0.02% NaN_3). The suspension was concentrated with a stirring Amicon cell equipped with a Millipore ultrafiltration membrane (100 kDA).

Modified isolation and purification of potato phosphorylase

In a modified isolation process 1 mM of 0.2 M PMSF stock solution in ethanol was added during the mincing process. The rest of the process was performed in the same way as in the previously described isolation process.

Activity assay for potato phopshorylase

3.9 mL of a solution containing G-1-P (100 mM) and G-7 (1 mM) was prepared in Tris buffer (Tris 100 mM, pH 6.7, 0.02% NaN_3), the pH was readjusted to 6.7 and the reaction was heated to 37 °C. 100 µL aliquot was taken as a zero point and analyzed according to the procedure described in the paragraph spectroscopic measurement of Pi. 100 µL phosphorylase suspension was added and mixed for 20 min. Another 100 µL aliquot was taken and processed according to the same procedure.

Unit definition for potato phosphorylase

1 unit is defined as the amount of phosphorylase suspension that can release 0.1 mg Pi per 3 minutes.

Unit definition of Sigma-Aldrich for phosphorylase b from rabbit muscle

One unit will form 1 µmole of α-D-glucose 1-phosphate from glycogen and orthophosphate in the presence of 5′-AMP, per min at pH 6.8 at 30 °C measured in a system containing phosphoglucomutase, NADP, and glucose 6-phosphate dehydrogenase. (One µM unit is equivalent to approx. 45 Cori units.)

Led by this unit definition, phosphorylase *b* suspension is standardly made to contain 200 U mL^{-1}.

2.2.3 Synthesis

Synthesis of maltoheptaose

500 g of β-cyclodextrin was dissolved in 2 L 0.01 M HCL and was refluxed for 2 h. The mixture was neutralized with 1 M NaOH, slowly cooled to room temperature, and stored overnight at 4 °C. By means of filtration the precipitated β-cyclodextrin was removed. 20 mL of p-xylene was added to the filtrate, mixed and heated to 60 °C in order to remove the remaining β-cyclodextrin that did not participate. After 1 h the filtrate was slowly cooled to room temperature and stored overnight at 4 °C. The resulting p-xylene/ β-cyclodextrin complex was removed via filtration and the filtrate was concentrated to 200 mL via rotary evaporation. The complete process with p-xylene was repeated and p-xylene/ β-cyclodextrin complex removed via filtration. The importance of removing β-cyclodextrin completely from G-7 is high, since it inhibits the phosphorylase reaction. [6] The filtrate was slowly precipitated in 2 L cold ethanol and dried in vacuum. The resulting G-7 appeared as a white powder.

Reaction course analysis

A mixture consisting of G-7 (1 mM), G-1-P (100 mM), phosphorylase *b* (100 µL of 200 U mL^{-1} suspention) or potato phosphorylase (100 µL of suspenstion), Dg GBE (0 for the synthesis of linear polysaccharides and 50 U mL^{-1} for the synthesis of branched polysaccharides), AMP (3.5 mM) and DTT (1.3 mM) dissolved in 4 mL buffer (Tris 100 mM, pH 6.7, 0.02% NaN_3) was incubated at 37 °C. The released Pi was followed at different times.

Synergetic Action of the Enzymes and appearance of the products

A mixture consisting of G-7 (0.3 mM), G-1-P (25 mM), phosphorylase *b* (0, 0.33 and 0.33 µM), Dg GBE (50, 0 and 50 U mL^{-1}), AMP (3.5 mM) and DTT (1.3 mM) dissolved in 2 mL buffer (Tris 100 mM, pH 7, 0.02% NaN_3) was incubated for 72 h at 37 °C. The released Pi was followed at different times.

2.3 Results and Discussion

In our work, due to the clarity linear polysaccharides and branched polysaccharides will be referred to as amylose and amylopectin analogs, respectively, irrespective of the phosphorylase used for the synthesis. We highlight that the same branching enzyme was used for the synthesis of all amylopectin analogs, whereas no branching enzyme was used for the synthesis of amylose analogs.

2.3.1 The effect of PMSF on the reactivity of potato phosphorylase

In order to ensure that the modification of potato phosphorylase isolation with the addition of proteases inhibitor PMSF is successful, phosphorylase was extracted with and without the addition of PMSF simultaneously.
If we compare the kinetics of both amylose and amylopectin analogs the syntheses catalyzed with potato phosphorylases isolated differently, (when PMSF is used for its isolation and when PMSF is not used for the isolation) we see that the polymerization of analogs synthesized with the potato phosphorylase isolated with PMSF is faster and yields products with higher DP than the one without. See Figures 2.1 (a) and (b).

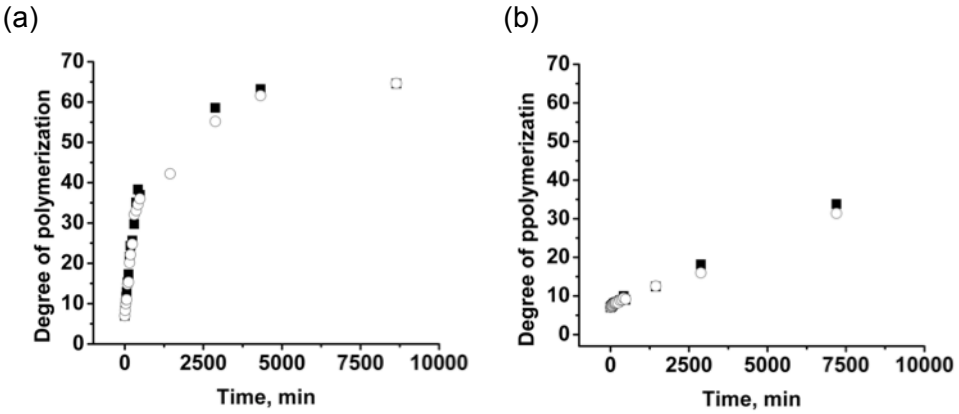

Figure 2.1. Kinetics of the synthesis of amylose analogs (white circle) and amylopectin analogs (black square) when **(a)** PMSF is added during the process of potato phosphorylase isolation and **(b)** when PMSF is not added during the process of potato phosphorylase isolation.

This implies that the PMSF has a significant effect of inhibition on the protease activity and diminishes the deactivation of the phosphorylase during the isolation process. A lower DP is reached when potato phosphorylase isolated without PMSF is used in the synthesis when compared to potato phosphorylase isolated with PMSF for the same reaction times. Even, if the reaction time is long enough (e.g., more than 350 h) similar DP cannot be reached. This suggests that most of the enzyme was destroyed by proteases and therefore the same volume of isolated phosphorylase solution without PMSF has less activity.
It is of no importance which analog was synthesized, amylose or amylopectin; the DP is equal at the same reaction time.
PMSF protects potato phosphorylase from proteases and therefore increases and prolongs its activity. The solution of phosphorylase isolated with PMSF stays active for months and the solution of phosphorylase isolated without PMSF does not. The observation of a high change in activity of potato phosphorylase upon the addition of PMSF implies that the majority of proteases in potato tubers from potatoes commercially available in stores in Groningen belong to the family of Serine Proteases (SP), since PMSF is a serine proteases inhibitor. This can support the fact that in recent years many new SP have been purified from a number of plant species, [20] where some were isolated from sweet potato. [21]

2.3.2 The effect of DTT on the activity of potato phosphorylase

In nature, enzymes are in a reducing environment; thus the sulfhydryl groups of enzymes are preserved in their reduced form. Reducing agents should be added to imitate *in vivo* conditions in order to maintain the enzyme's function, when enzymes are used as catalysts. [22] Therefore we tested the effect of the reducing agent DTT on the polymerization of amylopectin analogs when phosphorylase isolated with and without PMSF was used (see Figures 2.2 (a) and (b)).

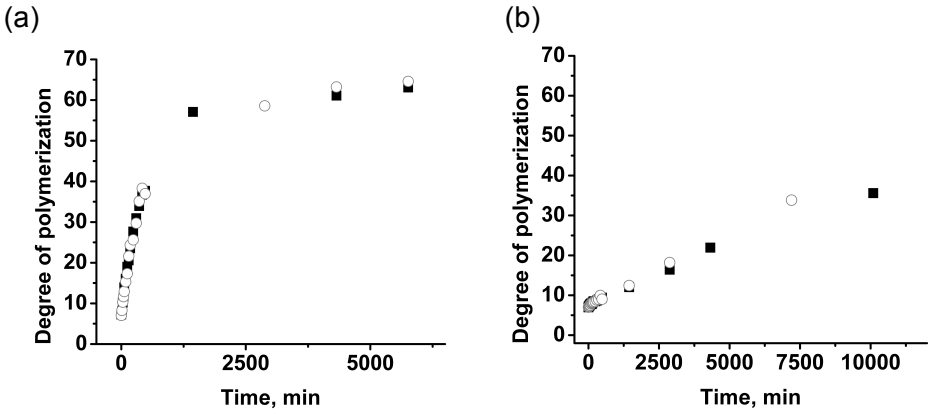

Figure 2.2. Kinetics of the synthesis of the amylopectin analogs when **(a)** PMSF is added to the potato phosphorylase isolation with DTT (black square) and without (white circle) and when **(b)** PMSF is not added to the potato phosphorylase isolation with DTT (black square) and without (white circle).

When we compared those results with the results from the section above it can be clearly seen that the effect of DTT on the DP is minimal. This is again the same for both analogs, amylose and amylopectin.

2.3.3 Synergetic Action of the Enzymes

An interesting publication with a similar synthesis as the one presented here, from Liu *et al*. [23] provoked us to combine Dg GBE with phosphorylase *b* from rabbit muscle instead of potato phosphorylase, even though phosphorylase *b* requires the use of an activator and reducing agent. (AMP and DTT respectively).
When phosphorylase *b* was introduced instead of potato phosphorylase it became obvious that the initial stage of the conversion is faster for the synthesis of amylopectin analogs, when compared to the synthesis of amylose analogs. Additionally, the conversion in total is higher for amylopectin analogs for a given reaction time (see Figure 2.3). It was already reported in literature that muscle phosphorylase acts more on branched acceptors than on the linear ones, [24, 25] which explains the higher conversion for the synthesis of amylopectin analogs in comparison to amylose analogs with phosphorylase *b*. The reaction mixture containing only Dg GBE was used as a blank reaction showing that Dg GBE alone does not produce any linear or branched polysaccharides, which is in accordance with the reaction mechanism of this enzyme. [26]

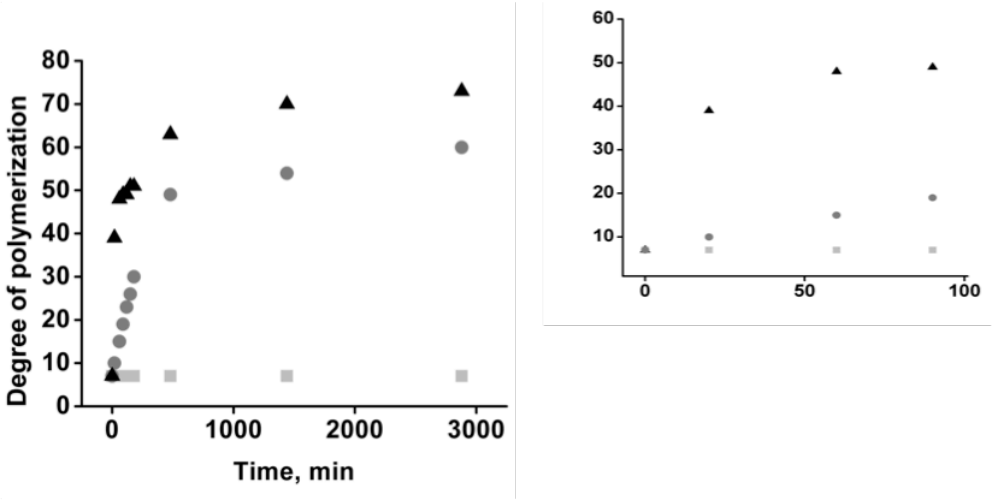

Figure 2.3. Kinetics of the reaction in which only Dg GBE was used (light grey square, phosphorylase blank reaction); where only phosphorylase *b* was used (dark grey circle); and kinetics for the tandem reaction (black triangle).

2.3.4 Comparison of the enzymatic synthesis of polysaccharides with different phosphorylases

To judge and decide which phosphorylase should be used for further work, the similarities/differences between the kinetics of the synthesis with all the enzyme used as catalysts are depicted in Figure 2.4.

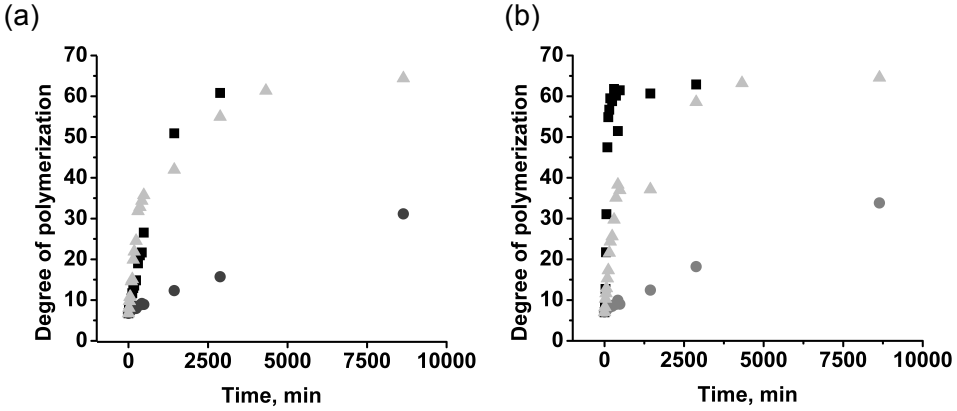

Figure 2.4. Kinetics of the synthesis of **(a)** amylose and **(b)** amylopectin analogs using different types and different isolation processes of phosphorylases: phosphorylase *b* (black square), potato phosphorylase without (dark grey circle) and with PMSF (light grey triangle).

The activities of the two different phosphorylase species (see experimental section) are determined in a different way, and in order to compare all tested phosphorylases we used the same volume of the enzyme suspensions. Nevertheless, phosphorylase *b* suspension was made from lyophilized enzyme in such a way to have the same number of units in 1 mL as the potato phosphorylase isolated with PMSF, regardles of the different determination of units. The potato phosphorylase isolated without PMSF, as explained in more detail above, had lower activity, hence less units in the same volume of suspenstion. It was used intentionally in that way to emphasize the benefits of the modified isolation process.

Figure 2.4 (a) shows that both the DP of amylose analogs and their dependence on the reaction time are very similar for the synthesis catalyzed by potato phosphorylase isolated with PMSF and the phosphorylase *b* (total activity in the reaction mixture 5 U mL^{-1}) catalyzed one, whereas

polysaccharides from the potato phosphorylase catalyzed reaction without PMSF do not reach the same DP, even in a few days reaction time, due to lower activity.
When it comes to the synthesis of amylopectin analogs there is a significant difference between the reactions catalyzed with different types of enzymes, which is nicely depicted in Figure 2.4 (b). DP reaches a maximum value already after a few hours when phosphorylase *b* is used. Interestingly, this synthesis is catalyzed in the same manner considering DP when much lower concentration of phosphorylase *b* is used (total activity in the reaction mixture 1.5 U mL^{-1}). When potato phosphorylase isolated with PMSF is used, the same DP is reached after some time. To no surprise, since the activity of the potato phosphorylase isolated without PMSF is the lowest, the same DP cannot be obtained.
When the amount of phosphorylase *b* normally used for the synthesis of amylopectin analogs is applied (total activity in the reaction mixture 1.5 U mL^{-1} instead of 5 U mL^{-1}) in the synthesis of amylose analogs the reaction barely starts. The lower threshold for phosphorylase *b* to catalyze the synthesis of amylose analogs is 2 U mL^{-1} but preferable higher concentration of enzyme is used in the synthesis. This is an additional evidence for the difference of potato phosphorylase and phosphorylase *b* in the affinity towards substrates (Synergetic action of the enzymes section).

2.3.5 Cleaner products?

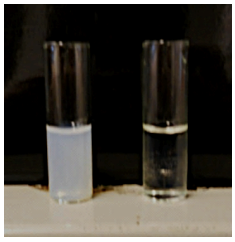

Figure 2.5. Visual appearance of amylopectin analogs solution in water when potato phosphorylase (on the left) and phosphorylase *b* (on the right) is used as a catalyst.

Figure 2.5 shows the difference between water solutions of amylopectin analogs synthesized with potato phosphorylase (on the left) and phosphorylase *b* (on the right). There is a clear difference in the appearance

of these solutions. The solution of amylopectin analogs synthesized with phosphorylase *b* is clear possibly due to the better solubility in water, the absence of contamination from the potato itself, or due to the combination of both. In order to find the difference in water solution appearance, ^{1}H-NMR and ATR FT-IR measurements were performed. The ^{1}H-NMR spectra showed no difference between the two measured samples, which can clearly be seen in Figure 2.6.

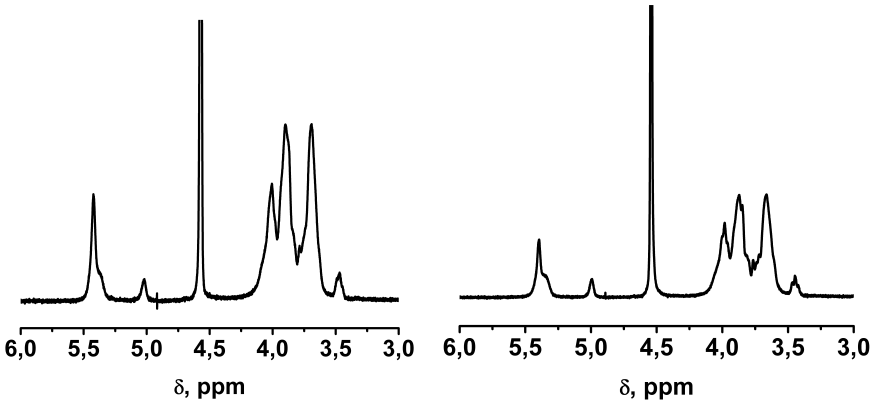

Figure 2.6. ^{1}H-NMR spectra of amylopectin analogs when potato phosphorylase (on the left) and phosphorylase *b* (on the right) is used as the catalyst; measured at 50 ˚C in D_2O.

When ATR FT-IR spectra were recorded, the difference between the two amylopectin analogs was clear for the samples with the identical ^{1}H-NMR spectra. Figure 2.7 illustrates ATR FT-IR spectra of pure G-7, the amylopectin analog synthesized with potato phosphorylase as the catalyst and the analog synthesized with phosphorylase *b* as the catalyst. On the one hand the spectrum when phosphorylase *b* is used as the catalyst is the same as the spectrum of pure G-7, which confirms the purity of the amylopectin analog. On the other hand the spectrum when potato phosphorylase is used as the catalyst differs from the spectrum of pure G-7 by the peak in the amide I' components band (1700 cm^{-1} - 1600 cm^{-1}) characteristic for enzymes, [27, 28] which confirms contamination of the amylopectin analog with the enzyme itself or similar compounds that result from the potatos.

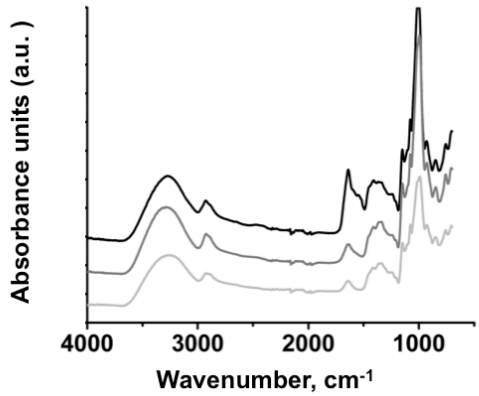

Figure 2.7. ATR-FTIR spectra of hyper branched amylose when potato phosphorylase (black) and phosphorylase *b* (grey) is used as a catalyst and G-7 (light grey) as the reference.

Besides the difference in the purity of the products, the DB was higher for the amylopectin analogs synthesized with phosphorylase *b* as the catalyst (see Table 2.1.). The value of 14% was positively surprising, since when potato phosphorylase and Dg GBE was used, the average DB usualy varied around the value of 11%. [1, 29] Higher DB could be due to the different catalytic mechanisms of phosphorylases or due to the possible partial protonation of the Dg GBE due to the addition of reducing agent. The various effects on the DB in the presented synthesis are elucidated in the following chapter.

Table 2.1. DP and DB values of amylopectin analogs.

catalyst	$\overline{DP_n}$ [a)]	**DB** [b)], **(%)**
potato phosphorylase	65	11
phosphorylase b	84	14

[a)]Determined via the colorimetric measurement of the liberated inorganic phosphate;
[b)]Determined via ^{1}H-NMR spectroscopy.

2.4 Conclusion

After comparing the catalytic activity of potato phosphorylase isolated with standard and modified methods, and monitoring the kinetics of the enzymatic synthesis of branched polysaccharides using those enzymes, we came to the conclusion that the suspension of potato phosphorylase isolated with the addition of PMSF has a higher activity and a longer lifetime. Additionally we noticed that a reducing agent, DTT in this case, has no effect on the potato phosphorylase.

Due to the affinity of phosphorylase *b* towards branched substrates, phosphorylase *b* is more appropriate for the synthesis of amylopectin analogs, since lower activity is needed and the higher degree of branching is achieved, when compared to the synthesis with potato phosphorylase.

Additionally, phosphorylase *b* and Dg GBE gave pure, water-soluble amylopectin analog.

For all the reasons mentioned above, phosphorylase *b* was our choice for further syntheses.

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CHAPTER 3

SYNTHESIS OF AMYLOPECTIN ANALOGS WITH TUNABLE DEGREE OF BRANCHING

An in vitro enzyme-catalyzed tandem reaction using the enzymes phosphorylase b from rabbit muscle and Deinococcus geothermalis glycogen branching enzyme (Dg GBE) to obtain amylopectin analogs with tunable degree of branching (2% ÷ 13%) is presented. The tunable degree of branching is obtained by varying the reaction conditions such as pH value, the choice of reducing agent and its concentration and reaction time. Linear amylose is formed by the phosphorylase-catalyzed propagation of glucose-1-phosphate while Dg GBE introduces branching points on the α-(1→6) position by relocating short oligosaccharide chains. Our results show that the best way to obtain different degrees of branching with this set of enzymes is by regulation of the reaction time.

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3.1 Introduction

Branched carbohydrates such as amylopectin differ to a large extent from their linear analogues, for instance they show differences in solubility, or rheological and mechanical properties. Many of the properties are necessary for food and non-food applications of starch and therefore the meticulous characterization of the molar mass, the type and the degree of branching of such materials is of great importance. However, good non-destructive characterization techniques for branched polysaccharides are rare. [1]

In order to establish an improved protocol for the characterization of branched polysaccharides well defined branched standards are required. Standards are materials that provide a reference to determine unknown concentrations or to calibrate analytical instruments.

In general the molecular size is not only dependent on the molar mass but also on the degree of branching as branching reduces the molecular size. [2]

The organic synthesis of well-defined (branched) polysaccharides such as chemical glycosylation is rather time-consuming and complicated; however, when enzymes are introduced as biocatalysts, the desired carbohydrates can be obtained easily. [3-5] Therefore, enzymatic polymerization can be utilized for the synthesis of branched polysaccharides as standards for improved characterization methods. [6]

For example, amylopectin analog, hyper-branched amylose can be synthesized *in vitro* by the combined action of phosphorylase (EC 2.4.1.1) and glycogen branching enzyme (EC 2.4.1.18). [7, 8] It was reported that branched polysaccharides with the degree of branching of 11% can be synthesized by the tandem action of potato phosphorylase and *Deinococcus geothermalis* glycogen branching enzyme (Dg GBE). [9]

However the reported synthesis has a couple of drawbacks. The potato phosphorylase formulation is not very pure and the resulting amylopectin analogs are contaminated with other carbohydrates as explained in detail in the previous chapter. Furthermore it was not possible to alter the degree of branching by using potato phosphorylase. It is known that phosphorylase *b* from rabbit muscle has more than 75% of the same active-site residues as potato phosphorylase and that they have many resemblances in their catalytic properties. [10] The action of phosphorylase *b* is well known in the literature and some are

commercially available enzymes. Therefore we decided to use phosphorylase *b* for the polymerization of amylopectin analogs. Additionally, the advantages when phosphorylase *b* is used have been explained in the second chapter of this thesis.

In cells, phoshorylase releases glucose-1-phosphate (G-1-P) from the non-reducing end of α-(1→4)-glucan chains. This reaction is reversible, hence when used *in vitro* with a high excess of G-1-P, phosphorylases catalyze the addition of glucose units to the chain and inorganic phosphate is released. It is well known that this synthesis requires the presence of a recognition unit suitable to start the polymerization, which is a co-substrate such as starch, glycogen or oligosaccharides. [11-13] The enzyme activity is controlled by the internal conversion between alternative structural states of the enzyme. [14] Phosphorylase *b* is the non-phosphorylated form of the enzyme and is activated by adenosine 5'-monophosphate (AMP). [15]

In nature, glycogens' side chains are introduced by glycogen branching enzyme. This enzyme is responsible for α-(1→6) branch points formation, by cutting α-(1→4) glycosidic linkage in the donor chain and transferring oligosaccharide from the nonreducing end to the α-(1→6) position. [16] It was previously shown that Dg GBE can be used to introduce a high degree of branching with an unusual side-chain distribution. [17]

Many factors such as temperature, pH value, water activity, ionic strength, chemicals such as reducing agents, etc. can affect enzymatic activity. For example, the thiol groups of some enzymes are voluntarily oxidized in air to disulfides. [18] Reducing agents such as glutathione (GSH), dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP) are often added to maintain these groups in the reduced state. [19]

By using phosphorylase *b* and Dg GBE we succeeded in obtaining amylopectin analogs in high purity. By varying the pH, the reaction time and the type and concentration of the reducing agent the molecular features of the branched polysaccharides (such as the degree of branching) could be adjusted opening the possibility to synthesize standards for the further development of characterization techniques.

3.2 Experimental

3.2.1 Materials and methods

α-D-glucose-1-phosphate disodium salt hydrate (G-1-P, Sigma-Aldrich), tris(hydroxylmethyl)amino metane (Tris, Sigma-Aldrich), 3-(N-morpholino)popanesulfonic acid (MOPS, Sigma), sodium citrate tribasic hydrate (Sigma-Aldrich), ammoniummolybdate (Sigma-Aldrich), potassium disulfite (Sigma), sodium sulfite (Merck), metol (Fluka), ammonium sulfate (Merck), sodium bisulfite (Acros), p-xylene (Merck), sodium azide (Merck), sodium acetate (Sigma-Aldrich), phenylmethanesulfonyl fluoride (PMSF, Sigma-Aldrich), phosphorylase *b* from rabbit muscle (Sigma-Aldrich), adenosine monophosphate (AMP, Sigma-Aldrich) and dithiothreitol (DTT, Sigma-Aldrich), isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma), Ni-NTA agarose column (QIAGEN), were used as bought. Glycogen branching enzyme from *Deinococcus geothermalis* (Dg GBE) was kindly provided by R.J. Leemhuis and L. Dijkhuizen or expressed and purified as stated later.

UV-Spectroscopy
^{1}HNMR Spectroscopy
Determination of the degree of polymerization
Determination of the degree of branching
Synthesis of Maltoheptaose
are explained in detail in Chapter 2.

3.2.2 Synthesis of amylopectin analogs

In order to establish a library of polyglucans with various degree of branching the methods described in literature by J. van der Vlist et al. [9] and W-C. Liu et al. [20] were adjusted accordingly. Different buffers Tris (100 mM, pH 7, 0.02% NaN_3); MOPS (50 mM, pH 7, 0.02% NaN_3) and reducing agents were used (DTT, GSH, TCEP). pH value of the reaction mixture, reducing agent concentration and the reaction time were varied whereas the concentration of G-1-P, G-7, AMP, phosphorylase *b* and Dg GBE were maintained constant if not stated differently. Excess G-1-P, AMP and reducing agents were removed by means of dialysis with 3500 MWCO tubes. Subsequently the samples were freeze-dried.

Effects of the reducing agent on the degree of branching

A mixture consisting of G-7 (0.3 mM), G-1-P (50 mM), phosphorylase *b*- (0.42 and 0.25 μM), Dg GBE (250 and 250 U mL^{-1}), AMP (3.5 mM) and DTT (0, 0.6, 1.3, 2.5, 5 mM) or TCEP (0, 0.6, 1.3, 2.5, 5 mM) or GSH (0, 0.6, 1.3, 2.5, 5 mM) dissolved in 2 mL buffer (Tris 100 mM, pH 7, 0.02% NaN_3) was incubated for 72 h at 37 °C.

Effects of pH on the degree of branching

A mixture consisting of G-7 (0.3 mM), G-1-P (50 mM), phosphorylase *b* (0.25 μM), Dg GBE (250 U mL^{-1}), AMP (3.5 mM) and DTT (1.3 mM) dissolved in 2 mL buffer (Tris 100 mM, pH 6, 6.5, 6.7, 7, 7.5, 8, 8.5 and 9 0.02% NaN_3) was incubated for 72 h at 37 °C.

Effects of time on the degree of branching

A mixture consisting of G-7 (0.3 mM), G-1-P (50 mM), phosphorylase *b* (0.42 μM), Dg GBE (250 U mL^{-1}), AMP (3.5 mM) and DTT (1.3 mM) dissolved in 2 mL buffer (Tris 100 mM, pH 7, 0.02% NaN_3) was incubated for 1, 2, 3, 4, 5, 6, 8, 24, 48, 72 and 100 h at 37 °C.

3.2.3 Overexpression of Dg GBE

Overexpression of the enzyme was achieved by overnight growth of *E. coli* (BL 21) containing the corresponding plasmid at 37 °C and 210 rpm in Luria-Bertani (LB) medium supplemented with 50 g mL^{-1} ampicillin. Protein was expressed successfully by induction with 1 mM IPTG at an optical density of ≈ 0.6 at 600 nm, followed by 4 h of additional growth. The cells were harvested by centrifugation (15 min at 9.000 x *g),* and the pellets were resuspended in 50 mL of 50 mM sodium phosphate buffer with pH of 8.0. The cells were disrupted using a French pressure cell press (25 kpsi). Cell debris was removed by ultracentrifugation (45 min at 40.000 rpm) and the supernatant was collected.
Purified proteins were obtained when the supernatant was applied on a 1 mL Ni-NTA agarose column. Subsequently the Ni-NTA agarose column was washed with 8 column volumes of 50 mM sodium phosphate buffer

(pH 8.0) containing 300 mM NaCl and 30 mM imidazole. Proteins were eluted from the column in 1 mL fractions with 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl and 250 mM imidazole. Fractions containing proteins were pooled and filtered using 50 kDa cut-off centrifugal concentrators (Millipore), at the same time buffer was exchanged from 50 mM sodium phosphate (pH 8.0) to 50 mM Tris 8.0.
Proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels that are shown in Figure 3.1. The conformation of molar mass was determined using a PageRuler Prestained Protein Ladder as a standard. The Dg GBE consists of 1,959 nucleotides and encodes an enzyme of 652 amino acids that has a predicted molecular mass of 74.4 kDa. [17]

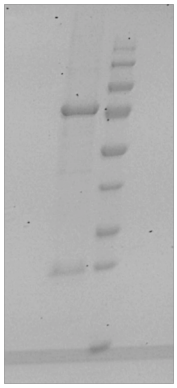

Figure 3.1. Proteins separated by SDS-PAGE.

3.3 Results and Discussion

3.3.1 Effect of the reducing agent on the degree of branching

Proteins are in a reducing environment when found in nature; hence sulfhydryl groups of enzymes are preserved in their reduced form. When dissolved under laboratory conditions, reducing agents must be added to imitate *in vivo* conditions in order to maintain the enzyme's function. [21] Three of the most common reducing agents in biochemistry were screened and the products of the enzymatic tandem polymerization were compared in order to see whether the degree of branching can be regulated by varying the concentration and the type of the reducing agent. A reference reaction was performed, without any reducing agent. The concentration of each reducing agent was varied from 0.6 mM to 5 mM for all agents.

Table 3.1. Degree of polymerization of amylopectin analogs synthetized with different reducing agent concentrations.

Reducing agent, (mM)	$\overline{\mathbf{DP_n}}$, **(0.25 μM phosphorylase b)**[a)]	$\overline{\mathbf{DP_n}}$, **(0.42 μM phosphorylase b)**[a)]
0	75	103
DTT (0.6)	79	90
DTT (1.3)	82	101
DTT (2.5)	73	85
DTT (5)	72	111
GSH (0.6)	82	132
GSH (1.3)	87	132
GSH (2.5)	88	117
GSH (5)	77	121
TCEP (0.6)	82	82
TCEP (1.3)	82	84
TCEP (2.5)	82	95
TCEP (5)	79	84

[a)]Determined via colorimetric measurement of the liberated inorganic phosphate;

Each of the agents follows a different trend, which can be seen in Figure 3.2. DTT has a tendency to increase the degree of branching in very small quantities reaching a maximum at 0.6 mM, with a continuous

decrease of the degree of branching at higher agent concentrations (Figure 3.2. (a)). TCEP, which is the strongest reducing agent in this research, has a constant trend for this range of concentrations by increasing the degree of branching to the same value with an exception for the concentration of 2.5 mM (Figure 3.2. (b)). The only natural antioxidant GSH continuously increases the degree of branching with an increase in concentration (Figure 3.2. (c)).

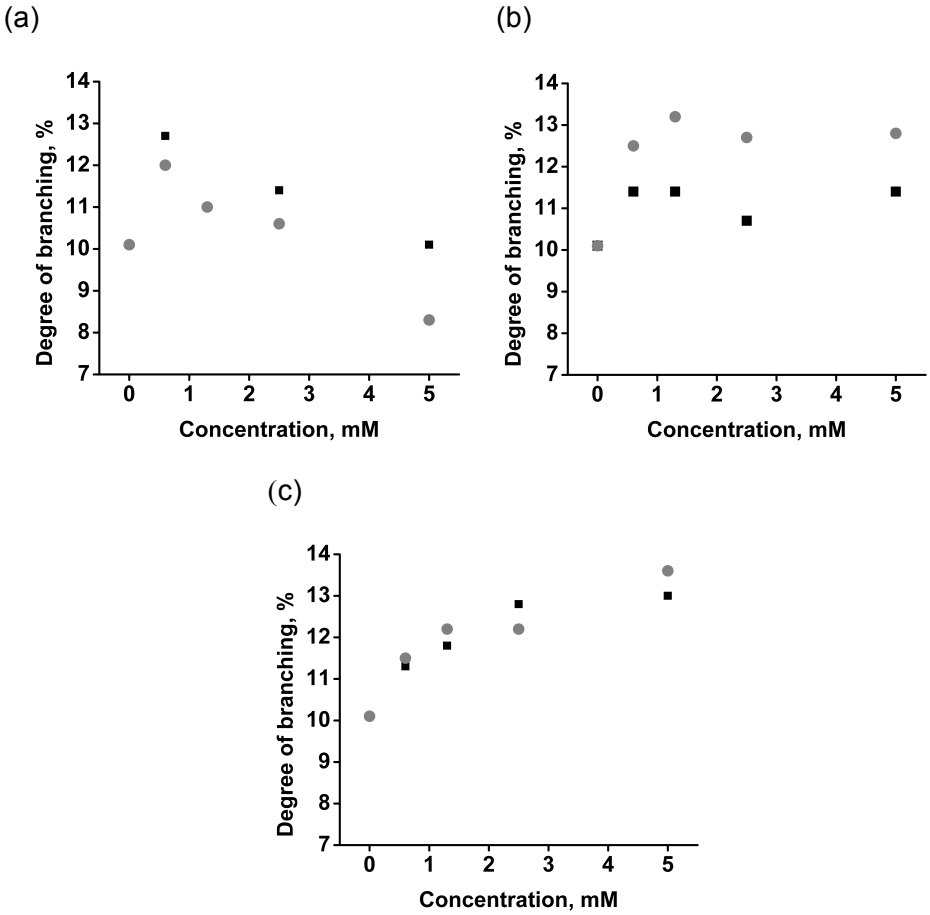

Figure 3.2. Degree of branching of amylopectin analogs synthesized by enzymatic polymerization with different reducing agents (**(a)** DTT; **(b)** TCEP, and **(c)** GSH) using different concentrations of the reducing agent and different enzyme concentrations (dark grey circle for 0,25 μM phosphorylase *b*, black square for 0,42 μM phosphorylase *b*)

During the experiments we also noticed that the reducing agents have an impact on the degree of polymerization. The addition of GSH, for both quantities of phosphorylase *b*, increases the degree of polymerization from 103 to 133. On the other hand the addition of TCEP reduces the degree of polymerization from 103 to 84 (see Table 3.1.).
Our work showed that the tandem reaction can be performed successfully without any reducing agent; however the type and the concentration of the reducing agent have an impact on the degree of polymerization and especially on the degree of branching. A small amount of the reducing agent increases the degree of branching. We assume that the partial protonation of the Dg GBE, together with the possibility of the protonation of the active site, results in the increase of the degree of branching as outlined below. In this experiment it becomes obvious that the degree of branching can be regulated by the amount of reducing agent. Moreover, it can be clearly seen that each of the selected agents follows a different trend for the dependence of the degree of branching and the agent's concentration, due to the different agent's properties. The phenomenon, that different reducing agents follow different trends is familiar in literature. [22] In the following work we use only DTT as the reducing agent, since it is the most commonly used one. Additionally we use only one concentration of phosphorylase *b* since there is no drastic difference in the results for different concentrations.

3.3.2 Effect of pH on the degree of branching

It is well known that enzymes are sensitive to the pH conditions of the solution. In order to choose the best buffer for further studies different buffers were used (Tris 100 mM, pH 7, 7.5 and 8, 0.02% NaN_3; MOPS 50 mM, pH 7, 0.02% NaN_3) for the synthesis of amylopectin analogs via the one-pot enzymatic tandem polymerization. The results were polymers of similar degree of polymerization and the same degree of branching for both buffers when compared at equal pH. In literature it was reported, that Tris buffer is best used when phosphorylase *b* from rabbit liver is used as an enzyme. [23] Consequently, Tris buffer was our choice for the future, since it can be used for a broader range of pH values.
The tandem polymerization reaction was effectively performed with potato phosphorylase at a pH of 7.0 at 37 °C according to literature. [9]

However, the optimum reaction conditions of phosphorylase *b* and Dg GBE are pH 6.9 to 7.4 at 38 °C [24] and pH 8.0 at 34 °C, respectively. By varying the pH value we noticed that by an increase of the pH value, starting with the lowest value of 6, the degree of branching increases and reaches the highest value of around 13% for a pH of 6.7 and afterwards starts to decrease continuously until the highest analyzed value of pH 9.

Table 3.2. Degree of polymerization of amylopectin analogs acquired at different pH values for the enzymatic polymerization of G-1-P, incubated for 72 h at 37 °C.

pH	$\overline{DP}_n$ [a)]
6.0	94
6.5	94
6.7	99
7.0	94
7.5	79
8.0	73
8.5	84
9.0	94

[a)]Determined via colorimetric measurement of the liberated inorganic phosphate;

The only decrease in degree of polymerization is observed between pH values of 7.5 and 8.5, whereas for the other values it stays constant (around 94) at equal reaction conditions (see Table 3.2). It becomes obvious that the degree of branching changes drastically with changing pH while the degree of polymerization in most cases stays the same. That brings us to the conclusion that Dg GBE is very pH sensitive whereas phosphorylase *b* is not. This is in accordance with literature - phosphorylase *b* is pH insensitivity above pH 6.4. [25] This effect allows us to synthesize amylopectin analogs with tunable degree of branching. The obtained degrees of branching at the different pH values after 22 h of reaction time at 37 °C are depicted in Figure 3.3.

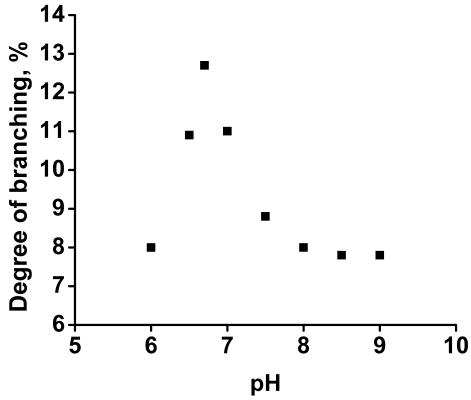

Figure 3.3. Degree of branching of polysaccharides synthetized at different pH values.

Our method enables tuning of the degree of branching (8% − 13%). The pH dependence of the degree of branching (activation of the Dg GBE) with addition of reducing agent is possible evidence of partial protonation of the active site of the Dg GBE. This is similar to the investigation of another group of enzymes reported in literature. [26] Furthermore, with the newly introduced enzyme, phosphorylase *b*, a slight modification of pH values, and the addition of the enzyme activator and the reducing agent, amylopectin analogs are synthesized with higher degree of branching than previously reported with potato phosphorylase. [9] This is possible due to the partial protonation of the Dg GBE, including the possible protonation of the active site.

3.3.3 Effect of time on the degree of branching

A satisfactory degree of polymerization is achieved after a short period of time (after 1h already 50% conversion of monomer is achieved), which provides the possibility to adjust the degree of branching via time regulation (see Table 3.3). This is due to the faster catalyzed polymerization of the linear chains by phosphorylase *b* compared to the cleavage and transfer of short oligosaccharides by Dg GBE in the tandem reaction. Therefore, the reaction can simply be terminated at the

required degree of polymerization and degree of branching, by heat treatment.

Table 3.3. The properties of amylopectin analogs synthesized at the different reaction time for the enzymatic polymerization of G-1-P, at pH 7 and 37 °C.

Time, h	$\frac{G-1-P}{G-7}$	$\overline{DP}_n$ [a)]	DB [b)], %
1	166.7	79	1.6
2	166.7	94	5.0
3	166.7	94	6.0
4	166.7	108	6.3
5	166.7	112	6.3
6	166.7	112	7.7
8	166.7	120	9.0
24	166.7	120	10.0
48	166.7	135	12.5
100	166.7	135	12.5
72	66.7	68	17.8

[a)]Determined via colorimetric measurement of the liberated inorganic phosphate;
[b)]Determined via ^{1}H-NMR spectroscopy.

When a lower degree of branching is required at a higher degree of polymerization, adjusting the ratio of G-1-P and G-7 can regulate it. [9] However, it was noticed that the degree of branching decreases with the increase in degree of polymerization for the same reaction conditions and different ratios of G-1-P and G-7. This is possibly due to steric hindrance, since the increase of concentration of Dg GBE does not increase the degree of branching. Too large numbers of molecules could prevent enzymes to reach the polysaccharide and catalyze both further polymerization and branching.

An exceptionally high degree of branching (around 18%) was only obtained for the smallest tested ratio of G-1-P and G-7 and the degree of polymerization of 68, other values varied from 11% to 13%.

It is clear from the data in Table 3.3 that the degree of branching increases with time when phosphorylase *b* is used in the tandem polymerization, in comparison to the constant degree of branching throughout the whole reaction time when potato phosphorylase is used.

[27] This is due to phosphorylase *b* affinity towards branched polysaccharides. [28, 29] The linear polymerization catalyzed by phosphorylase *b* is faster than the branching catalyzed by Dg GBE which means that time is the best mean to tune the degree of polymerization. Therefore, it is possible to synthesize a wide variety of amylopectin analogs with different degrees of branching and polymerization.

3.4 Conclusions

By an enzymatic tandem polymerization using phosphorylase *b* and Dg GBE various amylopectin analogs with tunable degree of branching and polymerization were polymerized.
During the kinetics analysis we concluded that potato phosphorylase and phosphorylase *b* have different affinities towards branched polysaccharides in comparison to linear ones. The tandem enzymatic polymerization is robust enough to perform without addition of any reducing agents; nevertheless both the degree of polymerization and the degree of branching appeared to be dependent on type and quantity of the reducing agent used in the polymerization.
Different pH values of the reaction mixtures, different reaction times, diverse reducing agents and their concentrations were tested and it was shown that products with different branching properties can be obtained by varying those parameters. It was shown that the best way to obtain different degrees of branching was by regulating the reaction time. The obtained polymers are pure and easily dissolve in water, which facilitates further analysis.

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CHAPTER 4

SIZE EXCLUSION CHROMATOGRAPHY WITH MULTI DETECTION IN COMBINATION WITH MALDI-TOF MS AS A TOOL FOR UNRAVELING THE MECHANISM OF THE ENZYMATIC POLYMERIZATION OF POLYSACCHARIDES

Determination of the size distributions of natural polysaccharides is a challenging task. More advantageous for characterization are well-defined synthetic (hyper)-branched polymers. In this study we concentrated on synthetic amylopectin analogs in order to obtain and compare all available data for different distributions and size dependence of molar masses. Two groups of well-defined synthetic amylopectin analogs were synthesized via an in vitro enzyme-catalyzed reaction using the enzyme phosphorylase b from rabbit muscle and Deinococcus geothermalis glycogen branching enzyme. Synthetic polysaccharides had tunable degree of branching (2% ÷ 13% determined via ^{1}H-NMR) and tunable degree of polymerization (30 ÷ 350 determined indirectly via UV spectrometry). The systems used for separation and characterization of branched polysaccharides were SEC-DMSO/LiBr and multi detection (refractive index detector, viscosity detector and multi angle light scattering detector); and SEC-water/0.02% NaN_3; and SEC-50mM $NaNO_3$ /0.02% NaN_3 and multi detection. Additionally the side chain length distribution of enzymatically debranched polysaccharides was investigated by MALDI-ToF MS analysis. With this combination of characterization techniques we were able not only to characterize the synthesized amylopectin analogs but also to solve parts of the molecular mechanism of their enzymatic polymerization. Moreover our materials showed potential to be standards in the field of natural polysaccharide characterization.

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4.1 Introduction

The most consumed polysaccharide in human diet is starch. It consists of two polymers, amylose and amylopectin; and possesses a simple chemical structure (glucose as a monomer) and a very complex molecular architecture (amylose - linear polymer and amylopectin - branched polymer). [1] Being excessively used in other branches of industry as well, the determination of the size distributions of starch and its components is one of the important steps in understanding its synthesis-molecular architecture-property relations. Determination of the size and mass distribution is one of the most challenging tasks in starch characterization. Difficulties occur due to distributions broadness and the structural complexity of starch, concerning variety in branch length and molecule size. [2, 3] The most popular and the most developed method for determination of the size distributions of starch and starch-like polymers is size-exclusion chromatography (SEC). [4-8]

The SEC separation of macromolecules is solely based on the hydrodynamic volume V_h of the characterized molecules. [9-12] IUPAC 's definition of $\mathrm{V_h}$ is "the volume of a hydrodynamically equivalent sphere". [13] For SEC separation, hydrodynamic volume is defined as:

$$\mathrm{V_h} = \frac{2}{5\mathrm{N_A}} [\bar{\eta}]_{\mathrm{w}}(\mathrm{V_h}) \bar{\mathrm{M}}_{\mathrm{n}}(\mathrm{V_h}) \qquad 4.1$$

where $\bar{\mathrm{M}}_{\mathrm{n}}(\mathrm{V_h})$ is the number average molar mass, $[\bar{\eta}]_{\mathrm{w}}(\mathrm{V_h})$ is the weight average intrinsic viscosity and $\mathrm{N_A}$ Avogadro's number. [11, 14, 15]

Molecular size distributions of natural (hyper)-branched polymers such as starch, are difficult to characterize for many reasons which include solubility problems, degradation during size separation, the broadness of size distribution, [16] shear scission, etc. [17] Well-defined synthetic (hyper)-branched polymers such as amylopectin analogs are more advantageous for characterization (better solubility in water, medium size molecules, insight into their degree of polymerization and the degree of branching before the analysis). Synthetic polysaccharides could help in understanding synthesis-molecular architecture-property relations of starches.

In this study, well-defined artificial amylose is synthesized via an *in vitro* enzyme-catalyzed reaction using the enzyme phosphorylase *b* from rabbit muscle. Well-defined amylopectin analogs are synthesized via an *in vitro* tandem reaction using the enzymes phosphorylase *b* and

Deinococcus geothermalis glycogen branching enzyme (Dg GBE) in order to obtain branched polyglucans with tunable degree of branching (2% ÷ 13%) and tunable degree of polymerization 30 ÷ 350 (see Chapter 1 Figure 1.6 and Chapter 3 Table 3.3). [18] The tunable degree of branching is obtained by regulation of the reaction time, [18] whereas the tunable degree of polymerization is obtained by regulation of the ratio between the monomer glucose-1-phosphate (G-1-P) and the primer maltoheptaose (G-7) as outlined in the previous chapter. [19] Linear amylose is formed by the phosphorylase-catalyzed propagation of G-1-P, [18, 20, 21] while Dg GBE introduces branching points on the α-(1 → 6) position by relocating short oligosaccharide chains. [22, 23]

The commonly used solvent for the characterization of starch-like polymers is DMSO with lithium salts or its combination with water for widely known reasons, such as good solvation without degradation at moderate temperatures, prevention of aggregation and adsorption of the material on the column. [16, 24] Nevertheless, due to high viscosity, working with DMSO can be tedious. In contemporary starch analysis water or buffers are used less, due to the limitations of solubility [4] and relatively austere conditions for sample preparation. [25-28] The best results can be obtained with the use of both systems and the comparison of the results.

In starch analysis the separation of linear amylose and branched amylopectin can occur as a problem. Separate analysis of each of the components can therefore be problematic and inaccurate. One way to avoid the problem of separation and the contamination of the components is to use synthetic individual components. Thus here we use well-defined synthetic amylose, and synthetic amylopectin (completely soluble in water). [18] Full separation of the two components when mixed together is presented and a complete analysis of the (hyper)branched standards in various solvents without austere sample preparation is possible.

Degradation due to shear scission of polymers with high molar mass in SEC analysis can occur both at the pore boundary and in the interstitial medium. [29] Analogy of branched polysaccharides shear scission and droplet shear suggests that shear scission is not that significant for polysaccharides with medium sizes (amylose and glycogen R_h< 120nm), analyzed with low flow rates. [17] However, for polymers such as amylopectin, there are no theoretical conditions at which shear scission

can be avoided. [7] Theoretically enzymatically synthesized well-defined amylopectin analogs with $\overline{M}_n$ lower than 50 000 g mol^{-1}, should avoid shear scission during their analysis by being in the medium size zone.
In this study we concentrate on starch like synthetic well-defined polysaccharides in order to obtain and compare all available data for different distributions and size dependence of molar masses. The systems used for separation and characterization of branched polysaccharides are SEC-DMSO/LiBr with PFG (polar modified silica) columns (used for the first time for this system and this type of polymers) and multi detection (refractive index detector, viscosity detector and multi angle light scattering detector); SEC-water/0.02% NaN_3; and SEC-50mM $NaNO_3$ /0.02% NaN_3 with Suprema (polyhydroxymethacrylate copolymer network) columns and multi detection.

4.2 Experimental

4.2.1 Materials and methods

All chemicals (α-D-glucose-1-phosphate disodium salt hydrate (G-1-P), (tris(hydroxymethyl)aminomethane (Tris), dithiothreitol (DTT), adenosine monophosphate (AMP), phosphorylase *b*, DMSO, NaN_3, $NaNO_3$) except LiBr (Fisher Scientific), maltoheptaose (G-7) and glycogen branching enzyme from *Deinococcus geothermalis* (Dg GBE), were purchased from Sigma-Aldrich and used without further purification. Dg GBE was kindly provided by R.J. Leemhuis and L. Dijkhuizen, whereas G-7 was synthesized as stated elsewhere. [19]
Appendix
Potato starch, wheat starch, corn and waxy corn starch were purchased from Sigma-Aldrich, Hylon VII from National Starch & Chemical Co, Eliane from Avebe food, whereas synthetic amylopectin analog was synthesized as explained in Chapter 3.

UV-Spectroscopy
^{1}HNMR Spectroscopy
Determination of the degree of polymerization
Determination of the degree of branching
are explained in detail in Chapter 2.

SEC of the polysaccharides using DMSO/LiBr as an eluent

The SEC system set-up (Agilent Technologies 1260 Infinity) from PSS (Mainz, Germany) consisted of an isocratic pump, auto sampler without temperature regulation, an online degasser, an inline 0.2 μm filter, a refractive index detector (G1362A 1260 RID Agilent Technologies), viscometer (ETA-2010 PSS, Mainz) and MALLS (SLD 7000 PSS, Mainz). WinGPC Unity software (PSS, Mainz) was used for data processing. The samples were injected with a flow rate of 0.5 mL min^{-1} into a PFG guard-column and three PFG SEC columns 100, 300 and 4000, which were also purchased from PSS. The columns were held at 80˚C and the detectors were held at 60˚C (Visco) and 45˚C (RI). A standard pullulan kit (PSS, Mainz, Germany) with molar masses from 342 to 805000 g mol^{-1}

was used to generate a universal calibration curve, in order to determine the hydrodynamic volume from the elution volume.

The values obtained in this work are the SEC weight distribution $w(\log V_h)$ (Equation 4.2) and the number distribution $N(V_h)$ (Equation 4.3), the size dependence of weight-average molar mass $\overline{M}_w(V_h)$ (determined from MALLS [30]) and number-average molar mass $\overline{M}_n(V_h)$ (Equation 4.4). In the following work data are presented in terms of hydrodynamic radii R_h, where $V_h = (\frac{4}{3}\pi R_h^3)$.

$$w\log(V_h) = S_{DRI}(V_e)\frac{d\tilde{V}e(V_h)}{d\log V_h} \quad 4.2.$$

$$N(V_h) = \frac{\eta_{sp}(V_e)}{V_h^2}\frac{d\tilde{V}e(V_h)}{d\log V_h} \quad 4.3.$$

$$\overline{M}_n(V_h) = \frac{5}{2}\frac{V_h N_A}{[\eta](V_e)} \quad 4.4.$$

In the following equations $S_{DRI}(V_e)$ represents the refractive index detector signal, $\frac{d\tilde{V}e(V_h)}{d\log V_h}$ the dependence of elution volume on hydrodynamic volume determined by the universal calibration curve, and $\eta_{sp}(V_e)$ the specific viscosity.

Both linear and branched samples were dissolved directly in previously prepared DMSO with the addition of LiBr in order to minimize interaction with the column and hydrogen bonding and to prevent retrogradation problems for linear samples,[31] at concentration of 2 g L^{-1}. The specific RI increment value, dn/dc for the well-defined branched polysaccharides in this system was taken to be the same as rice starch that consisted mainly of amylopectin, 0.0544 mL g^{-1}, [32] since they are amylopectin analogs. The specific RI increment value, dn/dc for the well-defined linear polysaccharides in this system was taken to be the same as amylose, 0.0689 mL g^{-1},[33] since they are amylose analogs. The samples were mixed for 3h at 80 ˚C and overnight at room temperature by a thermo shaker with 350 rph. All samples were filtered through 0.45 µm filters after shaking. Standards were dissolved in the same eluent at room temperature at 2 g L^{-1} concentration. The Mark-Houwink parameters for pullulan in this eluent at 80 ˚C were measured by PSS and are K=2.424 * 10^{-4} dL g^{-1} and α=0.68. [17] The specific RI increment value dn/dc was also measured by PSS and is 0.072. (Private communication with PSS)

The upper limit of R_h for the pullulan standards in this solvent in this study is 32 nm. There is no need for the extrapolation in this study since the synthetic branched polysaccharides have size below the upper limit of the calibration value.

SEC of the polysaccharides using aqueous (water and ammonium nitrate) eluent

The SEC system set-up was the same as for the DMSO analysis, with an inline 0.1 μm filter. The samples were injected with a flow rate of 0.5 mL min^{-1} into a Suprema pre-column and three Suprema SEC columns 100, 3000 and 3000, which were also purchased from PSS. The columns and the detectors were held at 50˚C. The same standard pullulan kit was used to determine the hydrodynamic volume from elution volume and the same calculation method was used to obtain desired values as in the DMSO system.
The branched samples were dissolved directly in previously prepared water or 50mM $NaNO_3$ with the addition of 0.02 % NaN_3 in both cases in order to minimize bacterial activity, at concentration of 2 g L^{-1}. The specific RI increment value, dn/dc for the well-defined branched polysaccharides, for the calculations, in this system was taken to be the same as pullulan 0.149 mL g^{-1}, since in literature values for amylopectin vary around this value. [26, 34] The samples were mixed overnight at room temperature by thermo shaker with 350 rph. All samples were filtered through 0.45 μm filters after shaking. Standards were dissolved in the same eluent at the room temperature at 2 g L^{-1} concentration.

Debranching of the polysaccharides [35]

6 mg of each sample was dissolved in 750 μl citrate buffer, (1 M, pH 4.0, 0.02% NaN_3) and debranched for 20 h at 40°C with 10 units of isoamylase from *Pseudomonas sp.* (Sigma-Aldrich). The debranched samples were dialyzed and freeze-dried. The distribution of the branches was analyzed with MALDI-ToF MS.

MALDI-ToF MS of the polysaccharides

Measurements were performed on a Voyager-DE PRO spectrometer in positive ion mode. 2,5-dihydroxybenzoic acid (DHB) was used as a matrix. The matrix solution was made by dissolving DHB (0.2 M) in a 1:1 v/v water/acetonitrile solution. Sample solution was prepared by dissolving the debranched sample in water R.O. (6 g L^{-1}). Sample and matrix were mixed in the ratio 2:1 or 1:1 v/v. 0.75 µL of the mixture was pipetted on the target and left for some time to dry.

4.2.2 Synthesis of well-defined linear and branched polysaccharides [18]

G-1-P was dissolved in Tris buffer (100 mM, pH 6.7, 0.02% NaN_3) containing G-7 (0.7 mM) as a primer, DTT (1.3 mM) as a reducing agent and AMP (3.5 mM) as phosphorylase *b* activator, and the pH was adjusted to 7. The polymerization was catalyzed by addition of rabbit-muscle phosphorylase *b* (1.5 U mL^{-1}) and the branching was initiated by Dg GBE (250 U mL^{-1}) at 37 ˚C Reaction time was 1, 3, 6, 9, 24, 72 h respectively to obtain the average degree of branching 1÷3, 3÷5, 5÷7, 7÷9, 9÷11, 11÷13. The concentration of G-1-P was 35÷420 mM to obtain the number-average degree of polymerization 30÷350. Termination was done by a 5 min heat-treatment. The samples were dialyzed to remove the excess of G-1-P, AMP and DTT. Afterwards, the samples were freeze-dried.

4.3 Results and Discussion

All analyzed samples were divided into two groups. The first group consisted of polymers synthesized at different reaction times in order to obtain various degrees of branching. The second group consisted of polymers synthesized with different ratios between the monomer and the primer (different monomer concentrations) in order to obtain different degrees of polymerization. Prior to all analysis, standard mixtures of completely linear (G-7 or synthetic amylose where Dg GBE was not used during the synthesis) and (hyper)-branched synthetic polysaccharides were analyzed on SEC systems to probe the separation.

4.3.1 Separation

Figure 4.1 (a) represents SEC weight distributions in DMSO, $w(\log V_h)$ plotted as a function of R_h for the highly branched synthetic polysaccharide ('amylopectin'-highly branched sample number 6.3 table 4.2), completely linear synthetic polysaccharide ('amylose', Dg GBE was not used during the synthesis), and a sample that contained a mixture (1:1) of both previously mentioned samples. Both samples had similar $\overline{DP_n}$ around 115. The presented remarkable separation shows that the use of PFG columns instead of most commonly used GRAM (polyester copolymer network) was a good choice. Furthermore the excellent separation will allow determination of molecular architecture of the samples containing unknown branched polysaccharides, linear polysaccharides, or both, by simple comparison of the size distributions of the samples with the size distributions of the standard mixtures for separation (known linear and branched sample mixed in a specific ratio) in the future. Figure 4.1 (b) represents the distributions in aqueous solvent of the same, branched sample; pure G-7; and a sample that consisted of both components. In this case G-7 was used instead of completely linear synthetic polysaccharide, since (synthetic) amylose with a degree of polymerization higher than 30 is not soluble in water at the temperatures used in this study. [36] It is clear that the separation between linear and branched polysaccharides is again very good and will be used later on for further explanations in this work. Additionally, the change in R_h in two different solvents indicates agglomeration, which will be discussed further on.

(a) (b)

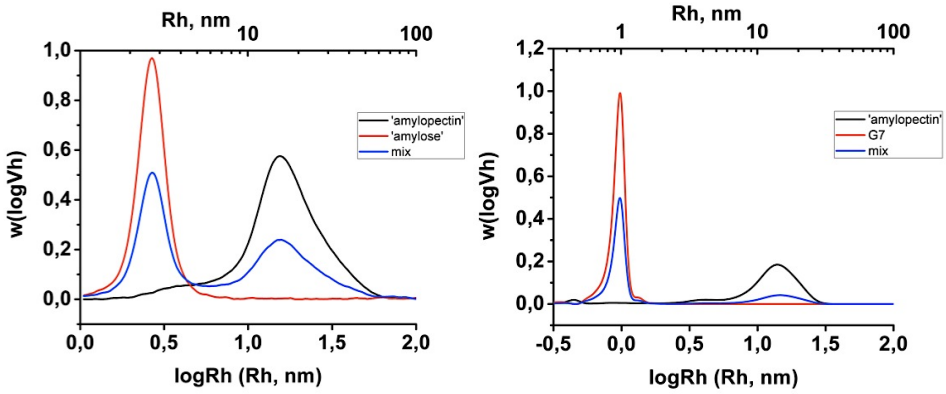

Figure 4.1. (a) SEC weight distributions $w(\log V_h)$ of highly branched (6.3-black line), linear (red line) and their mixture (mix-blue line) in DMSO **(b)** SEC weight distributions $w(\log V_h)$ of highly branched (6.3-black line), linear (G-7-red line) and their mixture (mix-blue line) in aqueous system.

4.3.2. SEC of the polysaccharides using DMSO/LiBr as an eluent

Different reaction time – different degree of branching

The enzymatically synthesized amylopectin analogs, that had the same starting ratio of the monomer (G-1-P) and the primer (G-7) but different reaction times (different degree of branching) during synthesis were tested using SEC with multi detection. Samples possessed similar degree of polymerization and different degree of branching. Prolonged cleavage and transfer of short oligosaccharides by Dg GBE compared to the rapidly catalyzed polymerization of the linear chains by phosphorylase *b* in the tandem reaction made different degree of branching possible. [18] The properties of the tested samples are shown in Table 4.1, containing number average degree of polymerization ($\overline{DP}_n$) determined indirectly from spectrometric determination of inorganic phosphate with modified Fiske and Subarrow method, [37] average degree of branching determined with ^{1}H-NMR, [19] and weight-average and number average molar masses ($\overline{M}_w$, $\overline{M}_n$) determined by SEC/RI/MALLS, for each sample. Additionally, some samples were tested with different

concentrations in order to test agglomeration of polymer chains. Even though no significant differences in MWDs were observed, the average molar mass slightly changed, which indicates that there is indeed agglomeration.

The $\overline{M}_n$ obtained with SEC was much higher than expected (agglomeration) and monotonically increased with the increase of the average degree of branching regardless of the average degree of polymerization. This rather unexpected result can be explained by the assumption that not all chains uniformly serve as branching donors and acceptors. The modified Fiske and Subarrow method used to determine the $\overline{DP}_n$ of the synthesized branched polysaccharides measures the concentration of converted monomer. Subsequently, $\overline{DP}_n$ is calculated by dividing converted monomer with the number of chains started (amount of maltoheptaose used). However, during the performed enzymatic polymerization some linear amylose chains that get created by the action of phosphorylase plainly serve as branch donors and therefore get completely consumed in the end. This results in fewer chains than the used primer sequences (maltoheptaose). Consequently, $\overline{DP}_n$ measured spectrophotometrically differed from $\overline{DP}_n$ measured by SEC measurements as only the later method could account for the decreased amount of chains and therefore measured the correct $\overline{DP}_n$.

Table 4.1. Properties of the enzymatically synthesized branched polysaccharides with different reaction times (different degree of branching)

S (Time) h	$\frac{G-1-P}{G-7}$	$\overline{DP_n}$ [a)]	DB [b)], %	$\bar{M}_n$, g mol^{-1} SEC DMSO	$\bar{M}_w$, g mol^{-1} SEC DMSO	$\bar{M}_n$, g mol^{-1} SEC water	$\bar{M}_w$, g mol^{-1} SEC water	$\bar{M}_n$, g mol^{-1} SEC $NaNO_3$	$\bar{M}_w$, g mol^{-1} SEC $NaNO_3$
1.4 (1)	300	96	3	1.26 x10^5	9.11x10^5	1.83 x10^4	5.13x10^5	1.68x10^4	4.01x10^5
2.4 (3)	300	110	7	2.34 x10^5	1.25x10^6	2.47 x10^4	6.76x10^5	9.78x10^3	9.48x10^5
3.4 (6)	300	145	7	5.31 x10^5	2.69x10^6	1.48 x10^5	7.99x10^5	8.92x10^4	1.05x10^6
4.4 (9)	300	204*	8	5.65 x10^5	3.25x10^6	3.38 x10^5	7.83x10^5	1.21x10^5	1.19x10^6
5.4 (24)	300	171*	10	1.12 x10^6	4.05x10^6	1.71 x10^5	9.03x10^5	2.36x10^5	1.15x10^6
6.4 (72)	300	192*	12	1.28 x10^6	4.41x10^6	3.46 x10^5	9.18x10^5	5.79x10^5	1.32x10^6

* The samples were synthesized separately, not in the same batch and therefore the value of $\overline{DP_n}$ differ between the samples after the equilibrium is reached (the tandem reaction follows kinetics in a way that in the initial stage the growth of the polymer is linear and levels off with time until the equilibrium state is reached [19]).

[a)] Determined via colorimetric measurement of the liberated inorganic phosphate;

[b)] Determined via ^{1}H-NMR spectroscopy.

The comparative SEC weight distributions $w(\log V_h)$ were plotted as a function of R_h, and are presented in Figure 4.2 (a). All samples that were synthesized longer than 1 h (DB> 3%) showed a clear bi-modal size distribution. With the increase of the average degree of branching, the low R_h peak decreased, whereas the high R_h peak increased. This indicates that the concentration of highly branched molecules increased and supports the theory mentioned above. During the tandem synthesis, phosphorylase catalyzed the formation of linear polysaccharides, which were affected by Dg GBE in two different ways. Short oligosaccharide were cleaved by Dg GBE from amylose chains and transferred to α-(1→6) position of a different amylose chain. However, amylose chains were not affected in both ways equally; therefore some donated oligosaccharides more than they accepted them.

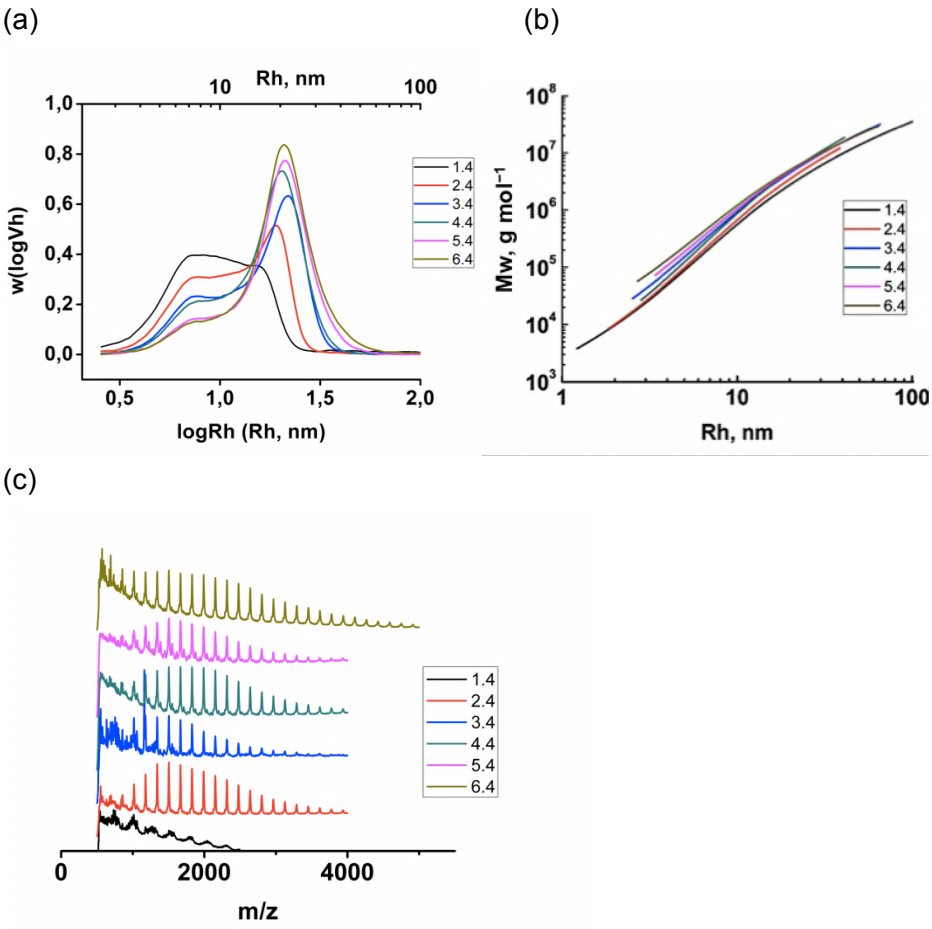

Figure 4.2. (a) SEC weight distributions $w(\log V_h)$ and **(b)** molar masses versus R_h of the enzymatically synthesized branched polysaccharides with different reaction time (different degree of branching). **(c)** MALDI-ToF MS spectra of the side-chain length distribution of debranched polysaccharides.
(sample codes from Table 4.1)

The number distribution $N(V_h)$ (see Figure 4.3 (a)), plotted as $\ln N(V_h)$ as a function of R_h, confirmed the two separate size areas (change of the slope at $R_h \sim$ 15 nm) for all samples with DB> 3%, with a sudden change in the slope. The $\overline{M}_n$ dependence on R_h (see Figure 4.3 (b)) did not show anything unexpected; it increased monotonically and showed that the number average mass became greater with the bigger particle size.

However the $\overline{M}_w$ dependence on R_h (Figure 4.2 (b)) showed that the $\overline{M}_w$ tended to overlap in the high R_h region for samples synthesized for 6 and more hours. This implies that after 6h the maximum degree of branching per molecule is reached, since the same molar masses have the same size. [8] The low R_h region showed an increase of the $\overline{M}_w$ with increase of the reaction time. This suggests that even though the concentration of molecules in low R_h region decreases (majority of the linear and less branched chains are transformed into highly branched ones by the action of Dg GBE and prolonged by phosphorylase) they become more branched with longer reaction time.

(a) (b)

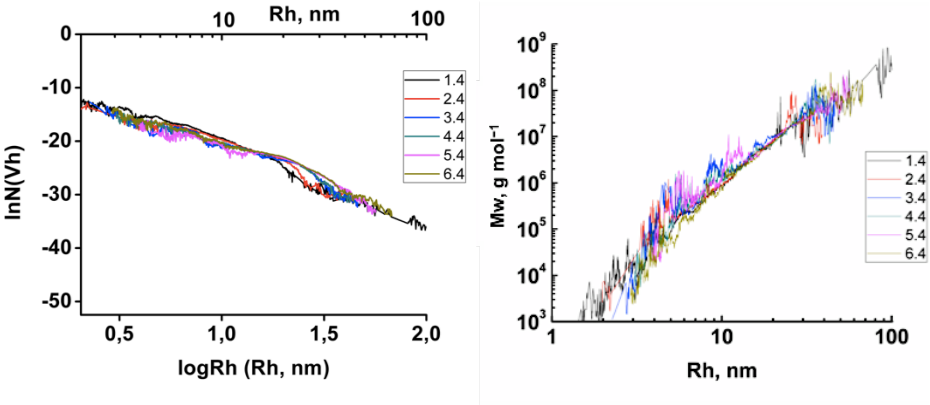

Figure 4.3. **(a)** Number distributions and **(b)** number-average molar masses of the enzymatically synthesized branched polysaccharides with different reaction time (different degree of branching).
(sample codes from Table 4.1)

Figure 4.2 (c) shows MALDI-ToF MS spectra of the debranched (with isoamylase at the α-(1→6) linkage) samples. The tested samples consisted of newly formed linear oligosaccharides after debranching. Due to the low degree of branching, the sample synthesized for 1 h (sample 1.4) showed almost no side-chains. With the increase of the reaction time to 6h (sample 3.4), the number of side chains increased with the most dominant chain length of 9 glucose units. With further increase of time (samples 4.4 ÷ 6.4) the chain length dominancy was suppressed, the intensity for various lengths became similar, and many lengths became

observable. The side-chain length distribution became broader and long side chains became visible. This gave us a reason to assume that the branching pattern in the high R_h region is not a typical amylopectin pattern with many short branches, [7] but resembles more that of for instance a polyethylene branching pattern with both long and short branches. [8] Regardless of the increase of the average degree of branching with time, in the high R_h region, individual highly branched chains stopped being further branched at a certain value, their concentration increased and their side-chains became longer. In the low R_h region, branching continued with time, even though the polymer concentration decreased in that area.

Different monomer concentration – different degree of polymerization

The amylopectin analogs, which had the same reaction time (72h) but a different starting $\frac{G-1-P}{G-7}$ ratio during the synthesis, were analyzed in the same way as outlined above. Expected results were uniformly highly branched polysaccharides with differences in molar mass. The properties are shown in Table 4.2. Unexpectedly, the average $\overline{M}_n$ decreased with the increase of the monomer concentration, and the average $\overline{M}_w$ fluctuated but in total also decreased. As previously mentioned the modified Fiske and Subarrow method measures monomer converted and $\overline{DP}_n$ is calculated by dividing this with the number of chains. Nevertheless, in this case we think that due to the increase of the concentration and therefore hindered diffusion some cleaved oligosaccharides did not reach the final destination (other polysaccharide chains) and became branches, but served as the new primers for the new linear chains. Consequently $\overline{DP}_n$ differed when calculated from SEC measurements and we again assume that the $\overline{M}_n$ obtained with SEC is the correct one.

Table 4.2. Properties of the enzymatically synthesized branched polysaccharides with different G-1-P/G-7 ratio (different degree of polymerization)

S	$\frac{G-1-P}{G-7}$	$\overline{DP}_n$ [a)]	DB[b)], %	$\overline{M}_n$, g mol^{-1} SEC DMSO	$\overline{M}_w$, g mol^{-1} SEC DMSO	$\overline{M}_n$, g mol^{-1} SEC water	$\overline{M}_w$, g mol^{-1} SEC water	$\overline{M}_n$, g mol^{-1} SEC $NaNO_3$	$\overline{M}_w$, g mol^{-1} SEC $NaNO_3$
6.1	50	43	12	$5.79x10^6$	$2.47x10^7$	$6.79x10^4$	$3.28x10^5$	$1.31x10^5$	$1.54x10^6$
6.2	100	59	12	$1.17x10^6$	$8.07x10^6$	$9.83x10^6$	$1.07x10^7$	$6.89x10^6$	$1.29x10^7$
6.3	200	112	12	$3.22x10^6$	$1.28x10^7$	$1.73x10^6$	$1.82x10^6$	$2.15x10^6$	$2.16x10^6$
6.4	300	192	12	$1.27x10^6$	$4.41x10^6$	$3.83x10^5$	$9.17x10^5$	$5.79x10^5$	$1.32x10^6$
6.5	400	221	11	$9.11x10^5$	$5.54x10^6$	$5.73x10^5$	$8.55x10^5$	$6.80x10^5$	$1.26x10^6$
6.6	500	290	10	$2.57x10^5$	$1.71x10^6$	$2.69x10^4$	$6.71x10^5$	$1.45x10^4$	$1.20x10^6$
6.7	600	342	10	$9.81x10^4$	$2.19x10^6$	$6.09x10^3$	$9.65x10^5$	$1.51x10^4$	$1.08x10^6$

[a)] Determined via colorimetric measurement of the liberated inorganic phosphate;
[b)] Determined via ^{1}H-NMR spectroscopy.

The comparative SEC weight distributions $w(\log V_h)$ are depicted in Figure 4.4 (a). Samples with higher ratios ($\frac{G-1-P}{G-7}$) than 100 showed a bi-modal size distribution. With the increase of the ratio, both the low R_h and the high R_h peak increased (low R_h very slowly). When the ratio became greater than 400, the low R_h peak continued to increase whereas the high R_h peak decreased, which indicates the concentration increase of the polymers with lower R_h. The peak maximum in the high R_h region shifted slightly towards lower R_h, with the increase of the ratio due to hindered diffusion (the synthesis of smaller molecules becomes preferable). This means that at that critical monomer concentration phosphorylase concentrates on two things: synthesis of linear chains; and prolongation of the newly created branches on the low-branched molecules (low R_h region). Surprisingly it does not concentrate on the prolongation of the branches on the highly branched molecules (high R_h region). The number distribution $N(V_h)$ (see Figure 4.5) confirmed two separate size areas (change of the slope at $R_h \sim 15$ nm) for all the samples with ratios greater than 50. Figures 4.4 (b) shows the dependence of $\overline{M}_w$ on R_h. In the low R_h region with the increase of the ratio a decrease of molar mass for the same R_h was visible, indicating that molecules in the low R_h region became less branched. In the high R_h region some of the samples again tended towards similar $\overline{M}_w$, for the same reasons as mentioned in the different reaction time group (different degree of branching).

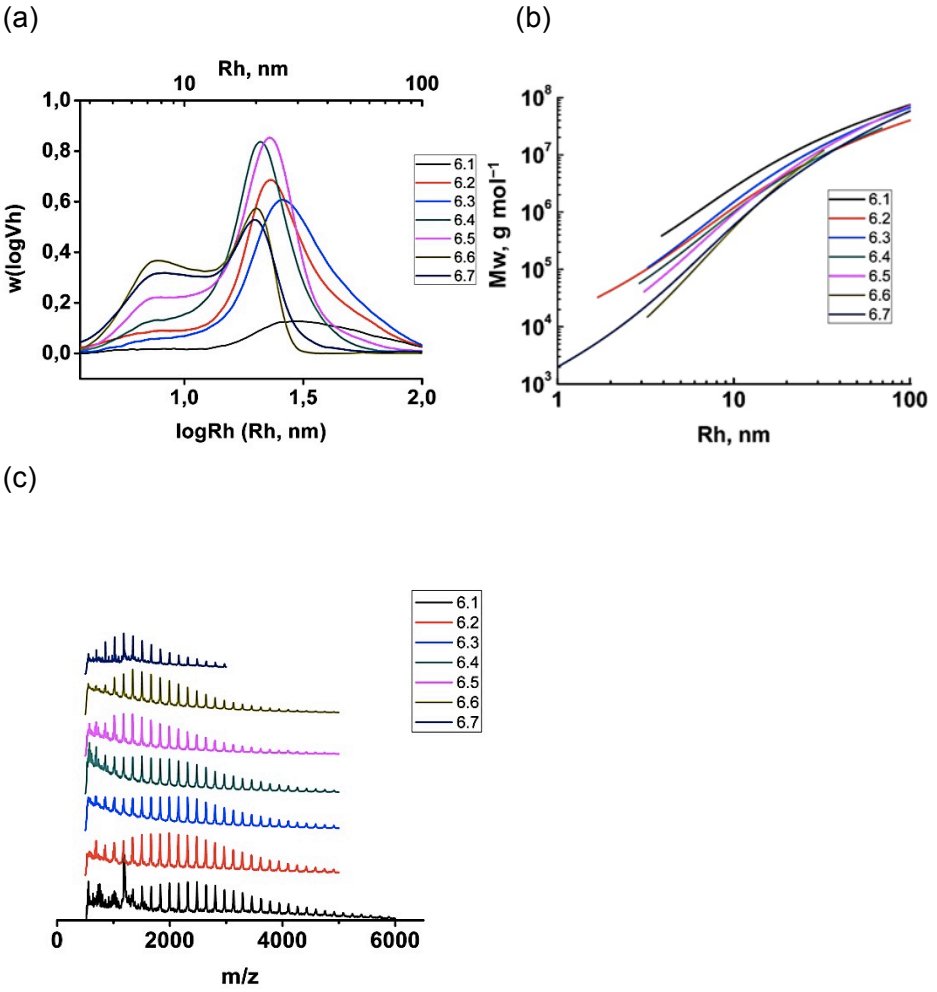

Figure 4.4 (a) SEC weight distributions $w(\log V_h)$ **(b)** molar masses versus R_h of the enzymatically synthesized branched polysaccharides with different monomer concentration (different degree of polymerization) and **(c)** MALDI-ToF MS spectra of the side-chain length distribution of debranched polysaccharides.
(sample codes from Table 4.2)

Figure 4.4 (c) shows MALDI-ToF MS spectra of the debranched samples. With the increase of the ratio, the most dominant chain length decreased from 14 to 7 glucose units. This suggests that due to hindered diffusion

not only smaller molecules are synthesized but also shorter chains are transferred from α(1→4) position to α(1→6) position. Since the side-chain length distribution became less broad and long side chains became less visible it can be assumed that the branching pattern in the high R_h region changes to a typical amylopectin pattern with many short branches. [7]

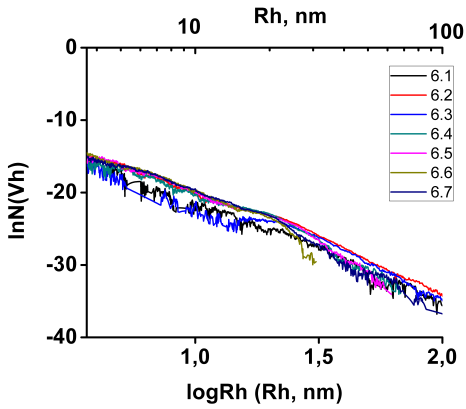

Figure 4.5 Number distributions of the enzymatically synthesized branched polysaccharides with different monomer concentration (different degree of polymerization).
(sample code from Table 4.2)

From these results, we propose that a critical number of molecules in the utilized enzymatic polymerization exists. When too many G-1-P molecules are present in the reaction the process of diffusion becomes hindered and the branching pattern changes; branched polysaccharides possess different branches characteristics. As it is clearly seen the higher the ratio becomes, for the same average degree of branching, the chains become shorter. After a critical point (the ratio of 400, sample code 6.5), the high R_h region starts to decrease and the low R_h region continues to increase as above mentioned. On the one hand the concentration of highly branched molecules decreases and their branches become shorter, and on the other hand the concentration of low branched molecules increases and they become less branched.

4.3.2 SEC of the polysaccharides using aqueous (water and sodium nitrate) eluent

Different reaction time (different degree of branching) and different monomer concentration (different degree of polymerization)

The same two groups of samples that were analyzed by SEC in DMSO were tested, in both water and aqueous 50 mM $NaNO_3$. Their properties are shown in Table 4.1 and Table 4.2. Additionally, all the samples were tested with lower concentrations, yet again in order to test agglomeration of the polymer chain. Once more the average molar mass slightly changed, which indicates that there is indeed agglomeration. The average molar masses were much lower than the ones with DMSO, in both aqueous systems. This suggests that even though agglomeration exists, it is much lower in the aqueous systems than in the DMSO one.
The comparative SEC weight distributions are presented in Figure 4.6 (a) and 4.6 (b) (water) and it can be clearly seen that the bi-modal distribution became multi-modal, with the major peak focus on the peak with bi-modal distribution, also visible in the DMSO analysis. The peak with bi-modal distribution confirmed all the conclusions made with DMSO analysis. In the different reaction time group (different degree of branching) the change of the bi-modal peak indicates that the concentration of highly branched molecules increases and confirms that some chains serve as the branch donors and some as the acceptors. In the different monomer concentration group (different degree of polymerization) the change of the bi-modal peak suggests that at a critical monomer concentration, diffusion becomes hindered due to a large number of molecules, and the synthesis of smaller molecules becomes preferable.

(a) (b)

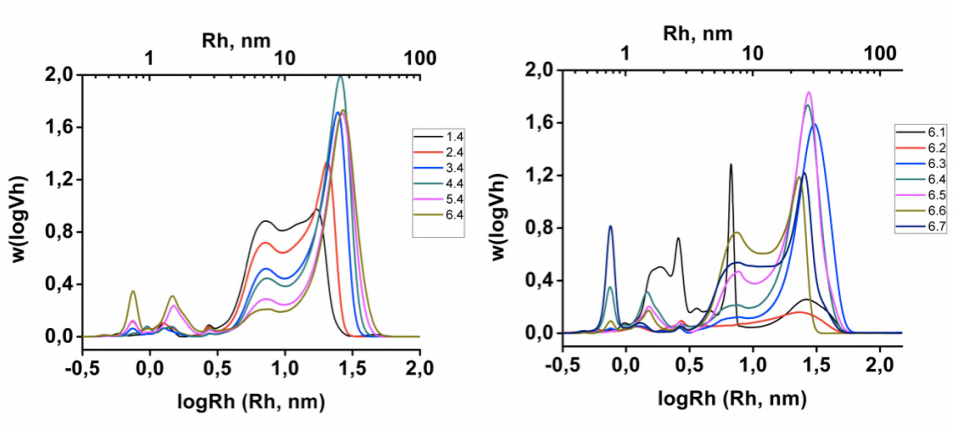

Figure 4.6 (a) SEC weight distributions $w(\log V_h)$ of the enzymatically synthesized branched polysaccharides with different reaction time (different degree of branching) and **(b)** of the enzymatically synthesized branched polysaccharides with different monomer concentration (different degree of polymerization) measured in water.
(sample codes from Tables 4.1 and 4.2)

(a) (b)

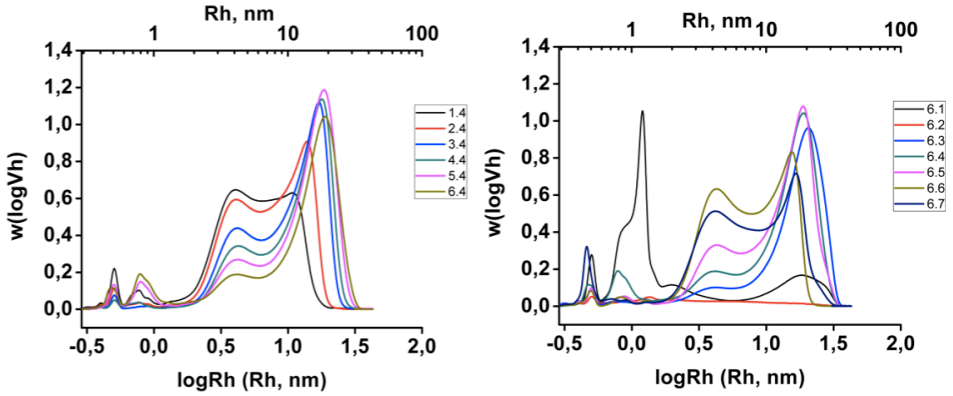

Figure 4.7 (a) SEC weight distributions $w(\log V_h)$ of the enzymatically synthesized branched polysaccharides with different reaction time (different degree of branching) and **(b)** of the enzymatically synthesized branched polysaccharides with different monomer concentration (different degree of polymerization) measured in aqueous 50 mM $NaNO_3$.
(sample codes from Tables 4.1 and 4.2)

The additional peaks at lower R_h when compared to the DMSO analysis could not be assigned to major interaction of the sample with the column, since the comparative SEC weight distributions in 50 mM $NaNO_3$ were completely the same (see Figures 4.7 (a) and 4.7 (b)). It can be assumed that due to better separation and less pronounced aggregation, additional low R_h peaks in the observed multi-modal distribution represent low molar mass, mostly linear polysaccharides (dialysis $15<\overline{DP_n}<30$ water insoluble). The low molecular, mostly linear chains are packed in between the highly branched chains due to high agglomeration and are therefore not visible in the DMSO analysis. In water they simply leach out of the agglomerates since some of the agglomerates are destroyed. The assumption of trapped chains being mostly linear is supported both by a very good separation of branched and linear polysaccharides with a higher $\overline{DP_n}$ in DMSO ($\overline{DP_n}$around 115) and of branched polysaccharides and G-7 in aqueous systems.

The dependence of $\overline{M}_w$ on R_h in both aqueous systems fully agrees with the DMSO analysis (see Figures 4.8 (a), 4.8 (b), 4.8 (c), 4.8 (d)). In the different reaction time group (different degree of branching) molecules in low R_h became more branched with longer reaction time and all the samples after 6h reached the maximum degree of branching per molecule in the high R_h region. In the different monomer concentration group (different degree of polymerization) molecules in the low R_h region became less branched with an increase of the ratio. In the high R_h region differences in the $\overline{M}_w$ became more visible than in the DMSO and from the critical monomer concentration (the ratio of 400, sample code 6.5) molecules started to be more branched again (the $\overline{M}_w$ starts to increase for the same size), which confirmed the change in the branching pattern suggested by MALDI-ToF MS analysis.

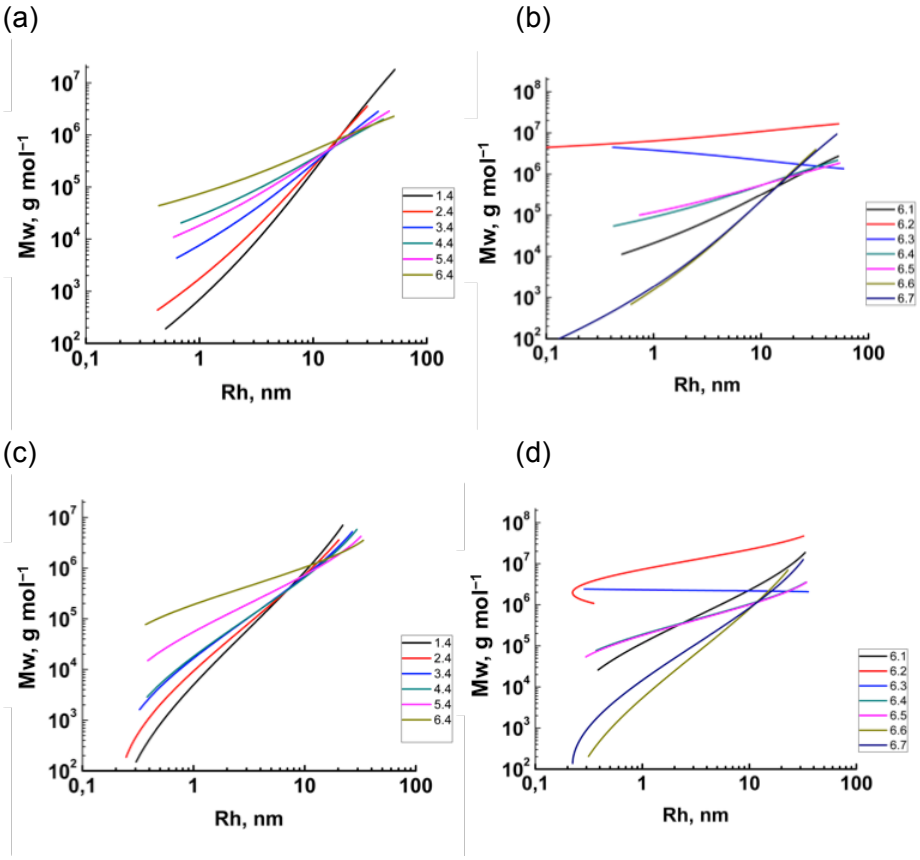

Figure 4.8 Molar masses versus R_h of the enzymatically synthesized branched polysaccharides with different reaction time (different degree of branching) **(a)** in water and **(c)** in 50 mM $NaNO_3$; and with different monomer concentration (different degree of polymerization) **(b)** in water and **(d)** in $NaNO_3$.

4.4 Conclusions

With the combination of characterization techniques we have solved parts of the molecular mechanism of the enzymatic polymerization of branched polysaccharides. With increase of the reaction time/degree of branching, the unexpected constant increase of the molar masses detected by SEC in comparison to indirect determination via spectrometric determination of inorganic phosphate can be explained by the hypothesis that some chains serve mostly as branch donors and some as acceptors during the one-pot enzymatic synthesis. With the increase of the monomer concentration/degree of polymerization, the unexpected constant decrease of the molar masses can be explained by hindered diffusion which changes the preferences of the enzymes and the fact that some of the cleaved short oligosaccharides serve as primers instead of becoming branches.

Furthermore our samples show an excellent potential to be standards for the field of starch characterization. In both analyzed groups, two size regions can be detected with both size and number distribution. On the one hand with the increase of the reaction time the high R_h region is more dominant and the side chains become longer, whereas the low R_h region decreases with time but the degree of branching increases in this area. In total the degree of branching increases with time, which is in accordance to ^{1}H-NMR data. It is interesting that the branching pattern in the high R_h region resembles more a polyethylene branching pattern than an amylopectin one. On the other hand with the increase of the monomer concentration the high R_h region's peak maximum transfers to slightly lower values and additionally at a critical point the low R_h region starts to increase, which means that the degree of branching per chain decreases even though the average degree of branching stays the same. It appears that Dg GBE prefers to transfer shorter oligosaccharides with the increase of the ratio and after a critical point the branching pattern in the high R_h region resembles more an amylopectin pattern.

Additionally, in this study, we showed that the use of PFG columns has the potential for a very good separation and analysis of linear and branched polysaccharides when DMSO/LiBr is used as a solvent. However, both water and 50mM $NaNO_3$ systems appear to be better solvents for analysis for synthetic branched polysaccharides; aggregation is less pronounced and the separation is better. Analysis in aqueous

systems fully agrees with the DMSO analysis and therefore strongly supports the presented conclusions.

4.5 Appendix

4.5.1 Analysis of debranched natural starches using SEC with multi detection (DMSO/LiBr and PFG columns)

One of the most complete characterizations of natural starches was achieved when the first two-dimensional structural distribution for starch was established in 2010, in which the weight distribution of whole starch molecules as a function of hydrodynamic size and of the hydrodynamic size of an individual branch was presented. [6] This technique combined size fractionation by preparative SEC, collection of size-separated fractions, enzymatic debranching of these fractions with isoamylase, and analysis of the branched and debranched fractions by analytical SEC with multiple detection in DMSO with GRAM (polyester copolymer network) columns. It was shown that the results of 1D branch chain length distribution and those from the 2D distribution (where unambiguous separation between amylopectin and amylose existed) gave acceptable agreement for the amylose content, with a moderate underestimation in the 1D measurements.
In this chapter, we showed that the use of PFG columns has the potential for a very good separation and analysis of linear and branched synthetic starch-like polysaccharides, when DMSO/LiBr is used as a solvent. The presented system with the synthetic polysaccharides helped in understanding synthesis-molecular architecture-property relations of starches. Additionally, this system due to previously shown good separation could be easily used for the determination of the amylose content of starch.
Therefore we wanted to expand this study, and test our system with natural starches. However, due to band broadening, shear scission and a large overlap between the small amylopectin molecules and the amylose, 1D whole-molecule size distribution significantly overestimates the amylose content. [6] To overcome this problem the size/molecular weight distribution of the branches can be obtained by enzymatic debranching of starch: each branch point is enzymatically cleaved by a standard technique. The resulting material is composed of entirely linear polymer chains, which we denote as linear starch, and avoids band broadening and shear scission due to reduced size. [16] Hence, we decided to test our system with this 1D branch chain length distribution method for amylose

content determination. Since the molecular architecture of starches is very important for their final use, additionally we check the size distribution of debranched starches and the dependence of molar mass on R_h. [27] These distributions affect viscosity and texture of the final products. [13] We also observed that the slight difference in molar mass for the same size of different starches does exist, even after debranching. This indicates an incomplete debranching with the used enzyme. Nevertheless, in this way it is possible to follow the difference in the structure of starches.

4.5.2 Results and discussion

A wide range of different starches with various amylose contents was analyzed (regular starches – potato, wheat or corn contain normally around 25 % of amylose, Hylon VII contains 70% of amylose, while the amylose content of Waxy starches is often less than 1%.). To compare with our previous results one synthetic amylopectin analog (SAA, reaction time 6h, G-1-P/G-7=500) was included and subjected to the same treatment and analysis as natural starches.
The starches were debranched by isoamylase for 16h and after purification analyzed by SEC with multi detection in DMSO/LiBr.
The distributions of the debranched starches are shown in Figure 4.9. When compared to literature, where different columns are used with the same solvent, the typical distribution patterns with slightly higher values for R_h can be seen. [6] As expected the distribution patterns of high amylopectin content starches (99% amylopectin - Eliane and Waxy corn) had a characteristic bimodal peak apportioned to the short amylopectin branches and no amylose peak. The bi-modal amylopectin fraction of the other starches and Eliane shows that Eliane has a higher ratio of long versus short chains. The synthetic amylopectin analog (SAA) had one characteristic peak in the area of short branches, which confirmed the conclusions made above in Chapter 4. To recall, when the ratio between G-1-P and G-7 is higher than 400, the branching pattern becomes similar to the glycogen branching pattern, with many short branches. [25] For the short amylopectin branches of the normal starches, a bimodal peak with R_h at log(R_h, nm) < 0.7 (corresponding approximately to DP < 100) is clearly visible. The separation from the amylose long chains peak (log(R_h, nm) > 0.7) is not 100% complete but clearly observable.

From these distributions, it is possible to calculate the ratio between amylose and amylopectin in the normal starches by comparing the areas under the curves (AUC) of the amylose peak to the total AUC of both amylopectin and amylose peaks if the peaks are separated or by simply checking a cumulative concentration distribution (corn and potato starch 30% amylose; Waxy and Eliane 5% amylose and wheat starch 55%, see Figure 4.10).

Overlap between the two areas is visible for Hylon, a high amylose content starch, which can be considered normal for this type of hybrid starches when debranched. [6] They consist mostly of amylose, therefore have much smaller molar masses than normal starches. Consequently it is worthless to attempt to separate the two areas with 1D chromatography, since the distribution of branches become wide and merges with amylose chains distribution. The combination of techniques that could provide slightly better separation in this case is fluorescent labeling/HPSEC. [26] Unfortunately, due to the small difference in the size between smaller hybrid amylose chains and debranched amylopectin one cannot yet expect a full separation.

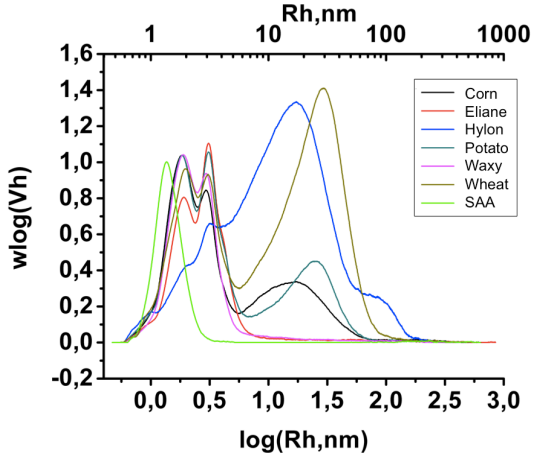

Figure 4.9. SEC weight distributions $w(\log V_h)$ of debranched starches

The only unexpected result was obtained in the case of wheat starch. It appeared that it has higher amylose content than expected (ca. 55% while below 30% is expected). As the separation between the amylopectin and amylose branch peaks is very good we suppose that in this case debranching was insufficient and/or low molar mass amylopectin was lost during purification.

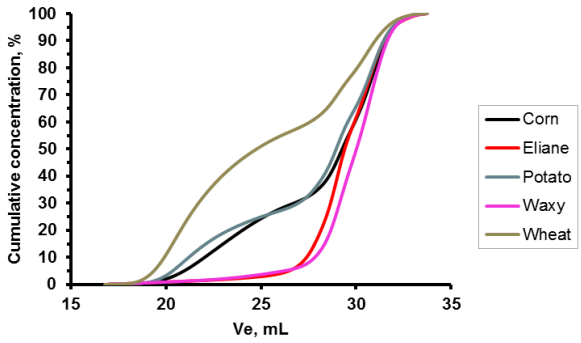

Figure 4.10. Cumulative concentration distributions of the debranched starches

Figure 4.11 represent the molar masses versus R_h of the debranched starches. For the molar mass distributions, in which the molecular size of one part of the debranched starches was too small for MALLS detection (in the case of Eliane, Waxy and the SAA – high amylopectin content) we represented the data collected via viscometer detection using universal calibration based on pullulan standards.

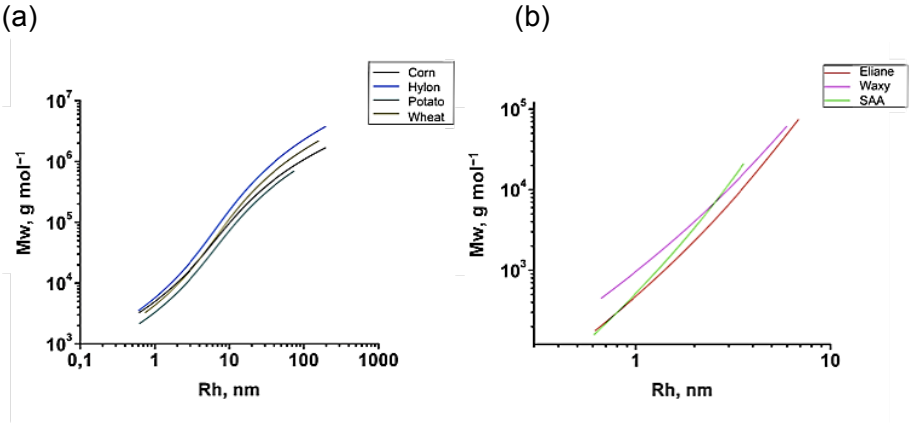

Figure 4.11. Molar mass measured with **(a)** LS and **(b)** viscometer (UC) versus R_h.

As expected, when amylopectin is debranched, the molar mass decreases drastically and the molar masses of the linear amylose parts reach much higher values since amylose has almost no branches, which

will be cut from the main chain by isoamylase. When compared with literature we see a very good agreement in both figures for the molar mass and R_h of the linear chains. [38] For instance, R_h of 5 nm and 20 nm correspond to molar masses of 2.5 10^4 and 2-3 10^5, respectively. Whereas molar masses of the branched polysaccharides with the same R_h are expected to be at least one magnitude bigger. A slight difference in molar mass for the same R_h is visible for the different starches. It is known that isoamylase is not able to debranch amylose completely [39] and therefore Hylon appears to be the most branched in the amylose area after debranching.

With these analyses we showed that SEC with multi detection, PFG (modified silica) columns and DMSO/LiBr can be used for the characterization of debranched natural starches. In future work PFG columns with different particle dimensions should be used, in order to investigate the whole- molecule size distribution and a 2D analysis. The amylose content can easily be determined. Additionally, the agreement for the relation size – molar mass of debranched parts was in a good agreement with literature.

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CHAPTER 5

PHYSICAL PROPERTIES AND STRUCTURE OF ENZYMATICALLY SYNTHESIZED AMYLOPECTIN ANALOGS

The mechanism of the enzymatic polymerization of amylopectin analogs with phosphorylase b and glycogen branching enzyme is very intriguing. In Chapter 4, size exclusion chromatography with multi detection of enzymatically synthesized amylopectin analogs in combination with matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS) analysis of enzymatically debranched analogs was used to solve parts of the molecular mechanism of analog's enzymatic polymerization.

In this work dynamic light scattering (DLS), atomic force and cryo transmission electron microscopy (AFM and TEM, respectively) were used to determine structural characteristics of the same analogs. The results were compared with size exclusion chromatography (SEC) analyses. The presented analyses in this work fully agreed with the recently made observations and confirmed the changes in the architecture of the synthesized polysaccharide due to the change of enzymatic polymerization mechanism.

Furthermore, we showed that the synthetic amylopectin analogs are stable to retrogradation at 4 °C if the main side chain length is no longer than 12 glucose units and that they have mostly fluid-like behavior in the form of 20% suspensions.

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5.1 Introduction

The relation of synthesis - molecular architecture- properties of starch and its components is very important as starch is the most consumed carbohydrate in human nutrition. However, it is very demanding to unravel this relation as starch is difficult to characterize partly due to solubility issues, stability, degradation during size separation, the broadness of size distribution, shear scission etc. [1, 2] During the last decades several approaches have been used in order to establish this relation. For instance, progress of biotechnology made it possible to produce tailor-made starches *in vivo* and to study the resulting properties. [3, 4] Furthermore, separation of the components of starch and their characteristics has been investigated intensively in the past. [5-8] Another approach is to study synthetic tailor-made analogs of starch components and to establish a detailed insight into the molecular architectures and properties via this route. We recently synthesized well-defined amylopectin analogues (hyper-branched amylose) successfully via an *in vitro* tandem reaction using the enzymes phosphorylase *b* and *Deinococcus geothermalis* glycogen branching enzyme (Dg GBE) as catalysts and glucose-1-phosphate (G-1-P) as a substrate (see Chapter 4). [9] The linear section of the amylopectin analogs were formed by the phosphorylase-catalyzed propagation of G-1-P. [10, 11] Branches at the α-(1 → 6) position were introduced by Dg GBE via relocation of short oligosaccharide chains from the α-(1 → 4) position (see Chapter 1 Figure 1.6). [12, 13] A tunable degree of polymerization was obtained by variation of the ratio between the substrate and the primer maltoheptaose (G-7), i.e. by the increase of the substrate concentration. [14] Furthermore, different average degrees of branching were achieved by the change of reaction time. [9]

The biocatalytic synthesis of (branched) polysaccharides was proven to be highly successful. [9, 14-16] Such synthetic polysaccharides can be used in different fields; such as hyperbranched glycoconjugates for drug delivery purposes or enzymatically synthesized glycogens as anti-tumor agents [15, 17] or as standards for new characterization protocols for branched polysaccharides. [17] The complete mechanism of the tandem enzymatic polymerization with phosphorylase and branching enzyme is not known yet; nevertheless, in Chapter 4, we unraveled parts of the mechanism by an in-depth characterization of synthetic amylopectin analogues using size exclusion chromatography (SEC) with multi detection in combination with the

analysis of enzymatically debranched molecules using matrix assisted laser desorption/Ionization time-of-flight mass spectrometry (MALDI-ToF MS). [17]
In Chapter 4 we established two groups of synthetic hyper-branched amylose - samples synthesized with different reaction time/degree of branching; and samples synthesized with different monomer concentration/degree of polymerization. We could show that with an increase of reaction time/degree of branching some of the linear or slightly branched amylose chains serve mainly as oligosaccharide donors for the new branches and some mainly as their acceptors during the synthesis. Simplified said, the product consists of less hyper-branched amylose molecules than primer sequences at the beginning of the reaction. Other interesting observations in this group of molecules were the existence of a two size regions (two distinct hydrodynamic radii R_h); a constant increase of the molar mass over time even after the consumption of the substrate stopped; long branches and a broad distribution of the branch lengths. Furthermore, regardless of the constant increase of the average degree of branching, branching in the high R_h area stops after a specific reaction time and in the low R_h area branching continues.
Furthermore, we have shown that in the second group of molecules - synthesized with different monomer concentration/degree of polymerization - with increasing monomer concentration some of the short oligosaccharides cleaved by Dg GBE can serve as primers instead of becoming branches during the synthesis. As a consequence, the product consists of more hyper-branched amylose molecules than primer sequences at the beginning of the reaction. In addition, a constant decrease of the molar mass regardless of the used amount of substrate and the appearance of much shorter side chains were observable. The existence of two size regions is present in this group as well. Additionally, regardless of the similar average degree of branching, branching in the low R_h area constantly decreases, whereas branching in the high R_h area is constant until a critical monomer concentration is used, at which the degree of branching starts increasing.
To shed more light on the relation of synthesis - molecular architecture-properties of starch we studied the physical properties of samples of the extreme ends from each of the two analyzed groups. The size of the molecules was assessed by dynamic light scattering (DLS), atomic force microscopy (AFM) and cryo transmission electron microscopy (cryo-TEM) measurements. The stability of the synthetic amylopectins at 4 °C was followed by differential scanning calorimetry (DSC) over 30 days.

Additionally dynamic rheological measurements are presented for the same chosen samples.

5.2 Experimental

5.2.1 Materials and methods

All chemicals (glucose-1-phosphate (G-1-P), tris(hydroxymethyl)-aminomethane (Tris), dithiothreitol (DTT), adenosine monophosphate (AMP), phosphorylase *b*, dimethyl sulfoxide (DMSO), NaN_3, $NaNO_3$) were purchased from Sigma-Aldrich except LiBr (Fisher Scientific) and used without further purification. Glycogen branching enzyme from *Deinococcus geothermalis* (Dg GBE) was kindly provided by R.J. Leemhuis and L. Dijkhuizen, whereas maltoheptaose (G-7) was synthesized as reported elsewhere. [14]

UV-Spectroscopy
^{1}HNMR Spectroscopy
Determination of the degree of polymerization
Determination of the degree of branching
are explained in detail in Chapter 2.

SEC and MALDI-ToF MS
MALDI-ToF measurements
are explained in detail in Chapter 4.

5.2.2 Synthesis of well-defined branched polysaccharides

The selected synthetic branched polysaccharides were synthesized as reported in literature. [9, 17] G-1-P was dissolved in Tris buffer (100 mM, pH 6.7, 0.02% NaN_3) containing G-7 (0.7 mM) as primer, DTT (1.3 mM) as reducing agent, and AMP (3.5 mM) as phosphorylase b activator, and the pH was adjusted to 7. The polymerization was catalyzed by addition of rabbit-muscle phosphorylase *b* (1.5 U mL^{-1}) and the branching was initiated by Dg GBE (250 U mL^{-1}) at 37 °C. The reaction time was 6 or 72 h, respectively, to obtain the average degree of branching 7 and 10−12. The concentration of G-1-P was 35−420 mM to obtain different number-average degrees of polymerization. Termination was done by a 5 min heat-treatment in a water bath. The samples were dialyzed to remove an excess of monomer, primer and reducing agent. Subsequently, the samples were freeze-dried. Estimation of the average degree of polymerization was performed by UV-spectrometry (indirectly via quantitative determination of the liberated

inorganic phosphate during the synthesis), [9] while the average degree of branching was determined by ^{1}H-NMR as explained in literature. [14] The polysaccharides used in this study are labeled 3.4 (reaction time 6 h, ratio between monomer and the primer 300, average degree of branching 7), 6.4 (reaction time 72 h, ratio between monomer and the primer 300, average degree of branching 12), 6.2 (reaction time 72 h, ratio between monomer and the primer 100, average degree of branching 12) and 6.7 (reaction time 72 h, ratio between monomer and the primer 600, average degree of branching 10).

5.2.3 DLS

DLS measurements were carried out at room temperature on a ALV CGS-3 goniometer set-up equipped with a JDSU laser model 1218-2 (wavelength λ_0 = 632.8 nm) and a ALV LSE-5005 multiple τ digital correlator. All measurements were performed in triplo and repeated on different days to check the repeatability of the procedure. DLS measurements were performed at suitable dilutions at angles between 30° and 150° with 20° interval. The refractive index of the solvent (water) is n_0 = 1.332: CONTIN algorithm was used to calculate the decay rates of the distribution functions. R_h was calculated from the diffusion coefficient, which was extrapolated to zero angle. In order to avoid dust, all samples were dissolved in filtered solvent (PTFE 0.2 µm filters Sartorius Stedim Biotech GmbH, Germany) and additionally filtered prior to analysis through PTFE 0.45 µm filters Sartorius Stedim Biotech GmbH, Germany.

5.2.4 AFM of well-defined branched polysaccharides

The sample was dissolved in filtered water, with a final concentration of 0.01 mg mL^{-1}; and deposited onto a mica substrate. Subsequently, the substrate with the sample was dried in air for 15 min at 60 °C. AFM images were recorded with a Multimode 8, controller V instrument operating in tapping mode with a SNL-10, A microcantilevers. During the measurements, the integral gain was 1.5V, proportional gain was 5V, and the scan rate was 1 Hz.

5.2.5 Cryo TEM of well-defined branched polysaccharides

Aqueous sample solutions were used with a concentration of 10 g L^{-1} for the cryo-TEM. Microscopy was carried out after rapid freezing of the samples in liquid ethane (Vitrobot, FEI, Endhoven, the Netherlands) and performed on a Philips CM12 transmission electron microscope operating at an accelerating voltage of 120 kV. Images are recorded on a Gatan slow-scan CCD camera under low-dose conditions.

5.2.6 DSC measurements of well-defined branched polysaccharides

The DSC measurements were carried out on a Perkin Elmer Pyris 1 DSC instrument. The retrogradation of well-defined branched polysaccharides was monitored at 70% water content. Samples were measured in previously weighed Stainless steel large volume pans (Perkin Elmer). After the addition of R. O. water the pans were sealed and reweighed. The sample pans were heated in an oven at 120 °C for 15 min. Cooled samples were stored for 1, 7 and 30 days at 4 °C prior to DSC analysis. The samples were heated from 0 to 120 °C at a heating rate of 10 °C min^{-1} using an empty sample pan as a reference. Each measurement was done in duplicate.

5.2.7 Rheological measurements

The rheological properties of 20 % suspensions from branched polysaccharide were determined with a Physica MCR 300 rheometer using a parallel plate geometry (diameter: 50 mm, gap width 1 mm) at 20 °C. As to the dynamic rheologycal measurements, gel characterization and linear viscoelastic region were determined by means of a strain sweep (0.1 % - 100 % strain at 1 rad s^{-1}). Subsequently, a frequency sweep (0.1 rad s^{-1} – 100 rad s^{-1}) was applied at 1% strain, which was well within the linear region.
Shear rate measurements were performed with the rate sweep program at a shear rate range of 0.1s^{-1} – 100 s^{-1}.

5.3 Results and discussion

5.3.1 DLS measurements

In Chapter 4 [17] we studied the dependence of the hydrodynamic radius R_h on the reaction parameters with SEC with multiple detection. To perform a more in-depth study we decided to additionally use DLS for the determination of R_h. The analyzed samples possessed a very low intrinsic viscosity (around 7 mL g^{-1}), which was independent of the molar mass. This is in agreement with research performed by Kajiura et al. [16] The low intrinsic viscosity suggests that the molecules behave according to hard spheres, as expected for glycogen-type molecules. [20] Only the sample with the longest side chains (6.2) had a slightly higher intrinsic viscosity compared to the others due to higher average chain length.[16]
Due to the low and molar mass independent intrinsic viscosity the calculation of R_h from the SEC data with multi detection could be deceptive. [17] Therefore, the calculation of R_h from DLS data via the Stokes–Einstein equation is much more reliable (R_h being dependent on the translational diffusion coefficient). [21] Consequently, the results acquired with DLS measurements were used for comparison and conformation of the estimations made previously with SEC – universal calibration (UC).
The R_h's of the tested samples are shown in table 1. The agreement of the R_h values measured with different techniques is quite good. For example if we compare the results for the sample 6.4 we can see a perfect agreement between DLS and SEC, both showing a R_h of 27nm. We should however bear in mind when comparing the results from those two techniques that one value results from a batch analysis (DLS) and the other one from column separation. Higher values from DLS measurements can be explained by the fact that bigger particles scatter more strongly and give a higher impact to the statistical value. [22] Additionally, as suggested by the SEC measurements previously performed (UC), DLS showed that with an increase of the reaction time/degree of branching the size of the polysaccharides increases and with an increase of the monomer concentration the size of the polysaccharides decreases.

5.3.2 Microscopy measurements

AFM images of the synthetic polysaccharide molecules are shown in Figure 5.1. The molecules being small in height (few nm) suggests that due to water

evaporation the molecules were oriented flat on the substrate. [16] AFM analysis suggests the existence of two major particle size regions and in consistence with all other techniques used so far an increase of the size of the particles with the increase of the reaction time/degree of branching. In addition, it becomes observable that with the increase of the monomer concentration the size of the polysaccharides decreases and particles become less uniform.

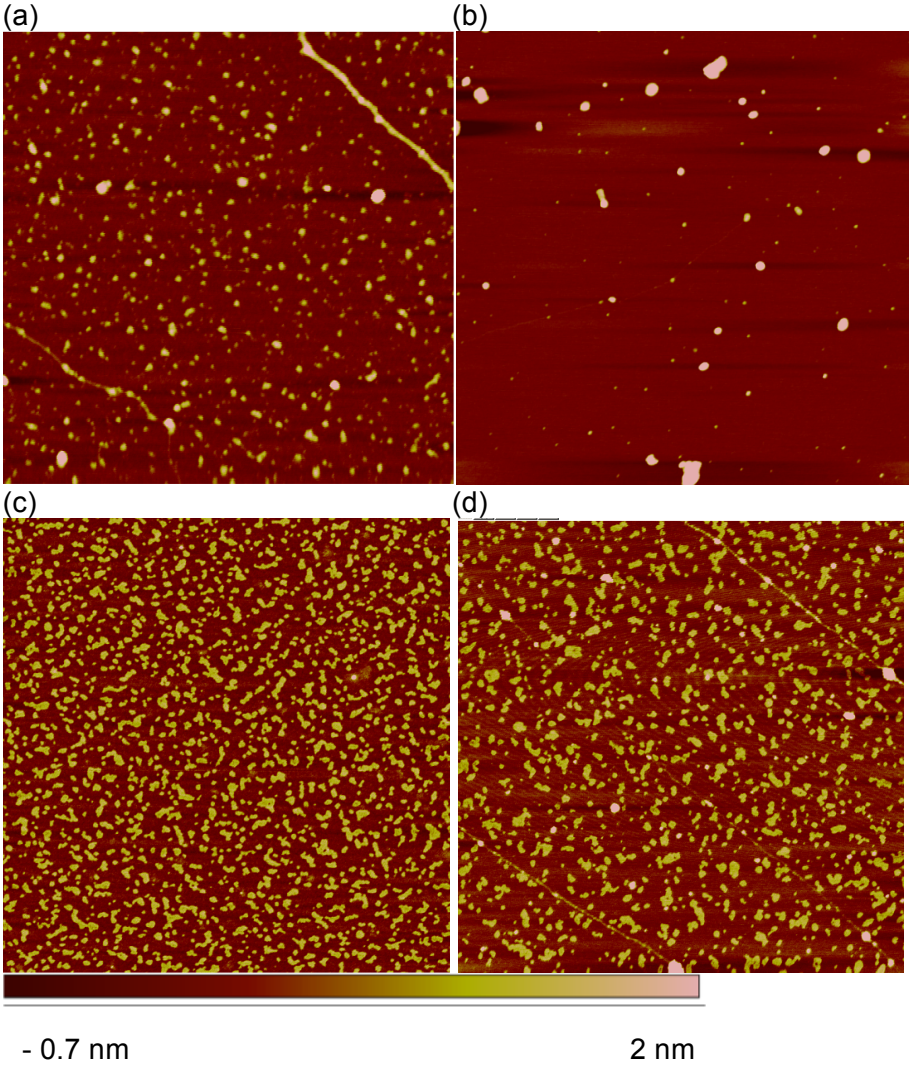

Figure 5.1. AFM images of synthetic polysaccharide **(a)** 3.4, **(b)** 6.4, **(c)** 6.2, **(d)** 6.7.

Figure 5.2 shows the cryo-TEM images of the synthetic polysaccharide molecules. In accordance with the AFM analysis, TEM suggests two particle size regions (low and high R_h). The radius measured with TEM is in agreement with the R_h measured by DLS or SEC with universal calibration curve. As already suggested by SEC analysis, [17] and the DLS measurements it is obvious that with an increase of the reaction time the radius in the high R_h region increases and with an increase of the monomer concentration the radius in the high R_h region decreases. Sample 6.2 is the only analyzed sample that formed rosette-like structures, already noticed in natural glycogens, but not in synthetic analogues. [16] It is known that β-particles of glycogen (20 – 40 nm diameter) can form larger α-particles by association (60 – 200 nm diameter) with rosette-like structure. [23] It is important to mention that the above mentioned sample had the highest molar mass, the longest side chains and the most broad distribution of side chains from all the samples investigated in this work. The formation of rosette-like particles could be due to the combination of those characteristics. Mostly the long side branches make the formation of α-particles possible, since β-particles are linked via α-(1→4) glycosidic linkages into α-particles. [24]

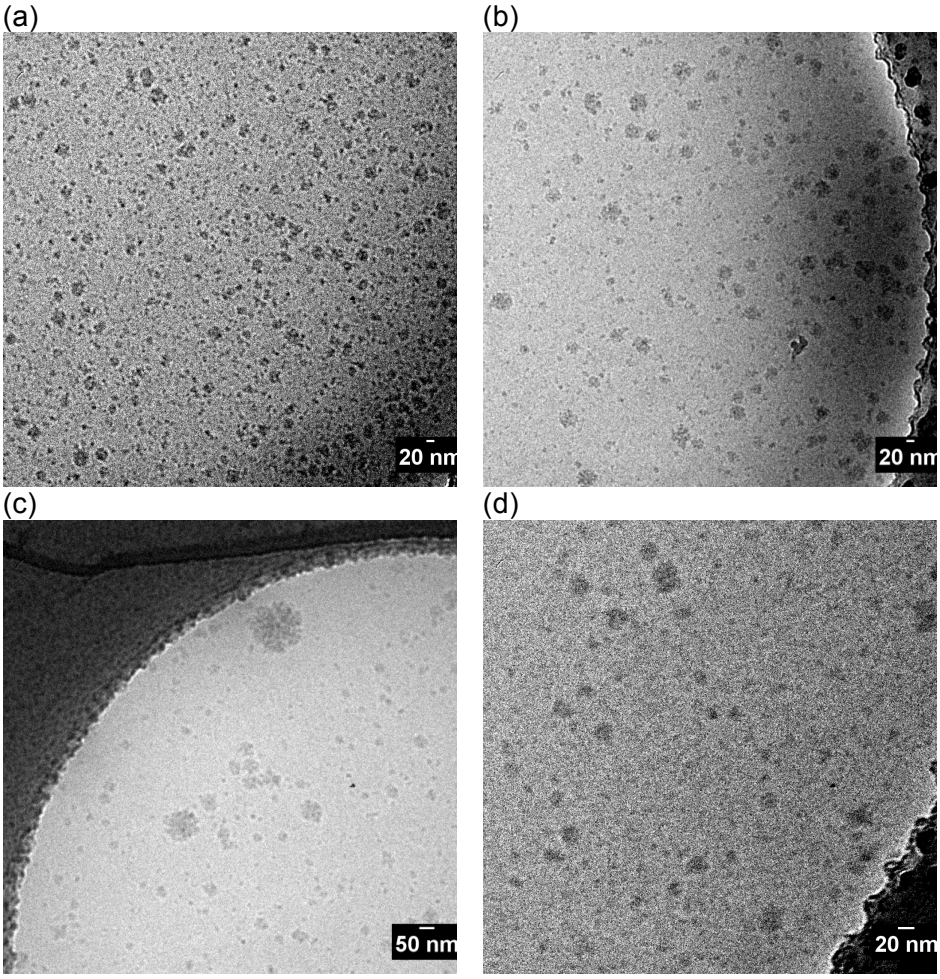

Figure 5.2. TEM images of synthetic polysaccharide **(a)** 3.4, **(b)** 6.4, **(c)** 6.2, **(d)** 6.7.

5.3.3 Retrogradation at 4°C followed by DSC

It is known for natural amylopectins that the process of retrogradation can take place in a few hours but also to over a couple of days depending on structure, concentration and temperature. When the retrogradation occurs, natural amylopectin gels reveal an endothermic peak in the region of 40 − 65°C. [5] External chains of amylopectin so called A chains are responsible for the formation of crystalline lamellas and reorganization of amylopectin during retrogradation. [25] The minimum chain length necessary for crystallization is supposed to be 10, but in the presence of longer chains short oligosaccharides can co-crystallize. [26] The rate of retrogradation is

dependent on the external chain lengths, being accelerated by longer chains and slowed down by the presence of short chains. [27-29]
The majority of the analyzed well defined branched polysaccharides stored at 4°C with excess water showed no presence of an endothermic peak in the monitored temperature area regardless of the storage time. This indicates that no or negligible retrogradation occurs even after 30 days.

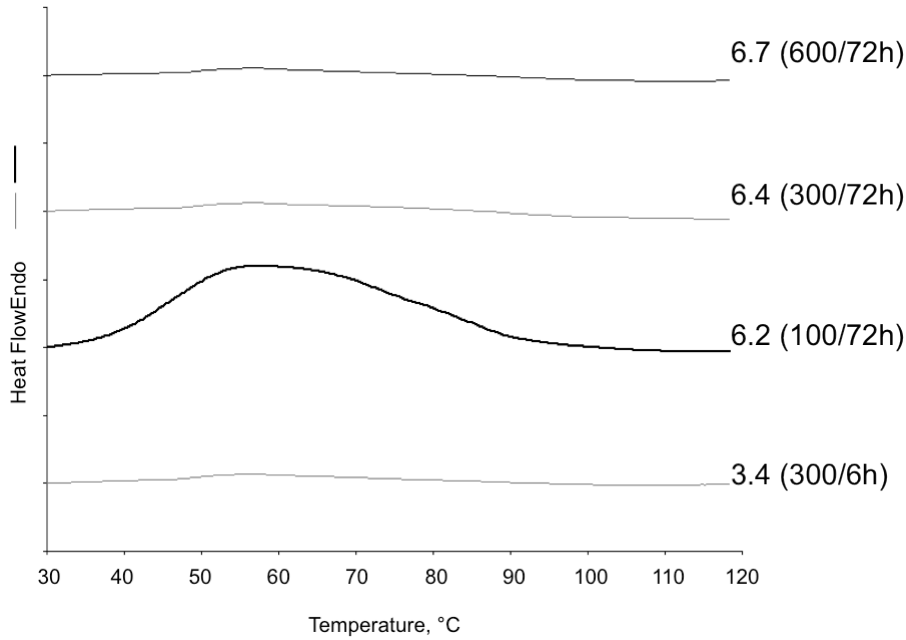

Figure 5.3. DSC melting endotherms for synthetic branched polysaccharides stored at 4°C for 30 days.

The sample 6.2 (lowest monomer concentration − longest reaction time used for the synthesis) was the only exception in this series. The endothermic peak observable in DSC was slightly broader than in the case of natural amylopectin gels (spanning 40 − 90°C) (Figure 5.3). The broadness of the peak, when compared to literature, could be due to the different sample amounts or measuring conditions used for the experiments. The stability towards retrogradation can be explained by the absence of amylose in the analyzed suspensions as it is known that starch suspensions in water that do not contain amylose show a lower tendency to retrogradation. [30] Additionally, retrogradation is dependent on the chain lengths of the branches. The only sample that did retrogradate had the main side chain length (the most dominant chain length in the distribution) of 12 glucose units

and a very broad distribution of side chains as analyzed by MALDI-ToF MS after debranching with isoamylase. [17] It is interesting that sample 6.4 (middle monomer concentration – longest reaction time used for the synthesis) which had two main side chain lengths (two dominant chain lengths) of 9 and 12 glucose units and broad distribution of side chains, [17] still showed no retrogradation. This indicates that even though the two samples (6.2 and 6.4) have similar average chain lengths they have different external chains, different outer architecture. This interesting observation is an additional support of the proposed mechanism for the enzymatic polymerization shown in Chapter 4. [17] Due to retrogradation, sample 6.2 had a different appearance when taken out of the cold; it was a gel-crystal-like white substance, while all other samples had the same liquid opaque appearance as the suspension made at room temperature.

5.3.4 Rheological study of 20% suspensions in water

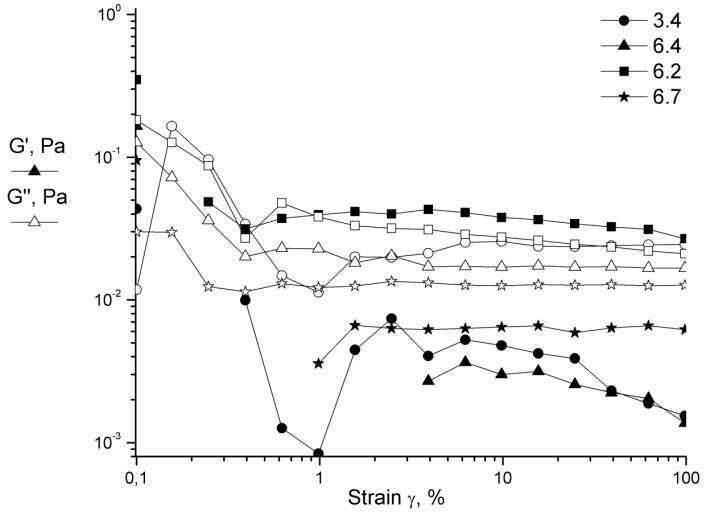

Figure 5.4. Strain sweep of 20% suspensions of branched polysaccharides in water.

Figure 5.4 shows the storage moduli G', and the loss moduli G", of all tested samples at 20% aqueous polymer concentration from the strain sweep analysis. Comparing all the samples at a given concentration, it became obvious that the only sample that possesses gel-like characteristics at this

concentration is sample 6.2 where G' > G". The frequency sweep measurement supported the strain sweep analysis. The obtained results are a clear indication that the hydrogen bonding between the big molecules is very weak and that no association occurs. This behavior is known in modified starches in which the presence of hydroxyethyl groups disrupts the hydrogen bonding and result in fluid-like behavior. [31, 32] The weakness of hydrogen bonding cannot be explained in our case by the presence of chemical groups, but with the architecture of the polysaccharides. The longer the external chains of the molecule are, the stronger the hydrogen bonding is between the molecules. During the rheological shear rate measurements we determined the viscosity of all the analyzed samples as well. The Newtonian zone was attainable for the 20% concentrations for all the samples, which can be clearly seen in Figure 5.5. The viscosities were extremely low, especially when compared to other polysaccharide solutions with that concentration. [32, 33]

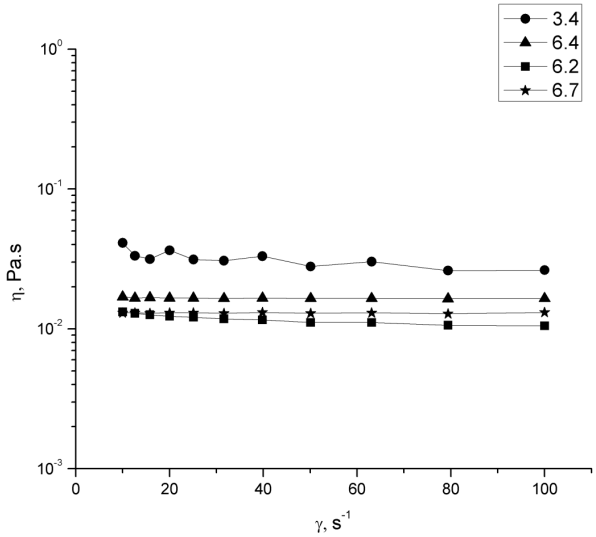

Figure 5.5. The viscosity functions of 20% suspensions of branched polysaccharides in water.

5.4 Conclusions

SEC analysis prior to the experiments in this work suggested the existence of two different particles concerning size; a region with high R_h and a region with low R_h. With an increase of the reaction time/degree of branching, we noticed an increase of the concentration in the high R_h region; constant increase of the average molar mass and a slight increase of the hydrodynamic radius. A combination of the presented techniques confirmed this behavior. By SEC measurements we noticed with an increase of the monomer concentration/degree of polymerization the increase of the concentration in both regions until the critical monomer concentration. When the critical monomer concentration is reached, the concentration in high R_h region starts to decrease. Unexpectedly, the molar mass and the hydrodynamic radius constantly decrease. The presented analyses in this work fully agree with the observations previously made by SEC.

In this work we also showed that the synthetic amylopectin is stable to retrogradation at 4 °C if the main side chain length is no longer than 12 glucose units and the polydispersity of the side chains is narrow. On the other hand we confirmed the changes in the architecture of the synthesized polysaccharide previously reported, [17] due to the change of enzymatic polymerization mechanism. Fluid-like behavior of polysaccharides at the analyzed concentrations, indicate the low strength of hydrogen bonding between the chains and the absence of strong aggregation, possible due to the short chains in total or the short external chains of the molecules.

In this chapter we showed interesting properties of the synthetic amylopectin, the effect of the synthesis conditions and the structure of the samples on the properties. The agreement of the particle size with different techniques such as DLS, SEC (UC) or cryo-TEM is very good. The stability towards retrogradation and low viscosity can be very interesting properties for the future application. Moreover these analyses support our previous research and suggestions made in it concerning the mechanism of the enzymatic polymerization of amylopectin analogs with phosphorylase *b* and Dg GBE.

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CHAPTER 6

CHARACTERIZATION OF ENZYMATICALLY SYNTHESIZED AMYLOPECTIN ANALOGS WITH ASYMMETRICAL FLOW FIELD FLOW FRACTIONATION COUPLED WITH QUASI-ELASTIC LIGHT SCATTERING AND MULTI ANGLE LASER LIGHT SCATTERING

Asymmetrical flow field flow fractionation (AF4), when coupled with multi-angle laser light scattering (MALLS), is a very powerful technique for determination of the macromolecular structure of high molar mass branched polysaccharides. AF4 is a size fractionation technique as size exclusion chromatography (SEC), nevertheless can overcome some crucial problems found in SEC analysis. Therefore, this chapter describes a detailed investigation of the macromolecular structure of the two groups of well-defined synthetic amylopectin analogs - synthesized via an in vitro enzyme-catalyzed reaction using the enzyme phosphorylase b from rabbit muscle and Deinococcus geothermalis glycogen branching enzyme (Dg GBE) previously analyzed by SEC with multi detection in Chapter 4. Size, molar mass distributions and structural data were studied by AF4 coupled with online quasi-elastic light scattering (QELS) and MALLS.

6.1 Introduction

"The most popular and the most developed method for determination of the size distributions of starch and starch-like polymers is size-exclusion chromatography (SEC)." Chapter 4
However, for good separation of branched and large polysaccharides in SEC the used columns and their limitations can be an obstacle. Currently used columns have low exclusion limits for extremely large polysaccharides, and lead to interactions of polysaccharides with the column material - especially in water; shear scission, and inevitable band broadening. [1, 2] A technique that can overcome these problems is asymmetrical flow field flow fractionation (AF4) that has already been used for the analysis of natural and synthetic branched polysaccharides. [3-5] For this reason, in order to verify the conclusions made via SEC analysis, we present in this chapter the analysis of enzymatically synthesized amylopectin analogs with AF4.
Field flow fractionation (FFF) is a group of high-resolution elution techniques that can separate nanoparticles in the 1-100 nm range and colloids up to 50 μm depending on the field used in the analysis. Size can be determined using either calibration with standards or FFF theory. The separation process is based on different physical interactions, depending on the type of FFF. There are three commercially available FFF techniques, thermal, sedimentation, and flow. The most widely used one is the flow FFF and it can be used in either the symmetrical or asymmetrical modes (FFFF or AF4, respectively). [6] The separation process in this type of FFF is based on the differences in the diffusion coefficients of the analyzed components. This implies that the separation depends on the size and shape of particles and molecules. Flow FFF is very similar to SEC, with the major difference is the lack of stationary phase. A typical AF4 setup can be seen in Figure 6.1. The biggest advantage of flow FFF when compared to SEC is that an upper molar mass size limit does not exist. However, when compared to column chromatography, it's drawback is the high sample dilution, that occurs in the channel during analysis. [7]

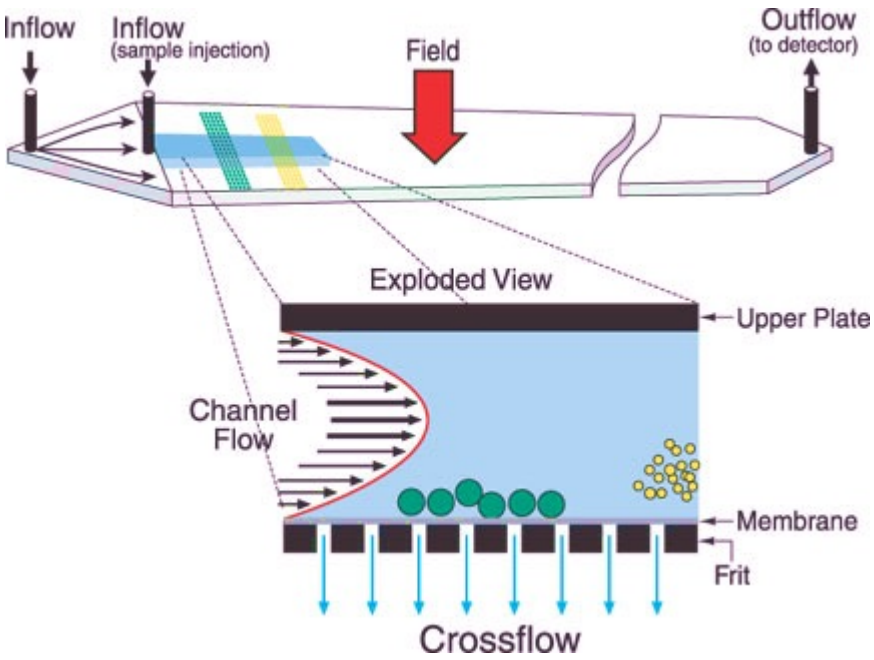

Figure 6.1. Schematic of the AF4 channel. http://www.wyatt.com/theory/theory/how-asymmetric-field-flow-fractionation-afff-theory-works.html

Fractionation occurs in a thin channel that is made using a polyester spacer (350 μm in our case) encased by one porous block (a frit). The laminar flow that carries the sample to the detectors through the system creates a parabolic flow-velocity profile across the channel. A fluid, which is perpendicularly applied is called a cross flow and drives particles against the lower, accumulation wall. The accumulation wall consists of a semi-permeable membrane on top of the lower stainless steel frit. The cross flow continuously drives particles against the wall, while Brownian diffusion pushes particles away from the accumulation wall into higher velocity flows. Due to the two opposing forces smaller particles interact with the faster part of the parabolic flow, and are eluted more quickly from the channel. [8] A typical separation so called normal separation (the desired one in the case of separation for macromolecules, by opposition to big particles higher than 1 μm size) is schematically shown in Figure 6.2.

Three successive phases occur in an AF4 experiment starting with an injection/relaxation/focusing of the sample, followed by elution and

finished with backflushing. During the first phase, the flow enters the channel via the inlet, whereas the only outlet is through the membrane. Firstly, in this phase, the sample is injected into the system (A). Secondly the sample is concentrated near the channel entrance (at the position determined by the counteracting flows, where the axial velocity is zero), in a focusing step (B). Sample components move to their respective equilibrium height above the membrane. Finally the separation occurs during the elution phase, in which the flow enters the channel via the inlet, but leaves both through the membrane (creating the crossflow) and the channels outlet. The carrier flow is laminar in the channel; hence the carrying speed of the sample is associated to the samples distance from the membrane wall (C). The smaller particles are driven easier by diffusion into the faster part of the flow, and are eluted first (D).

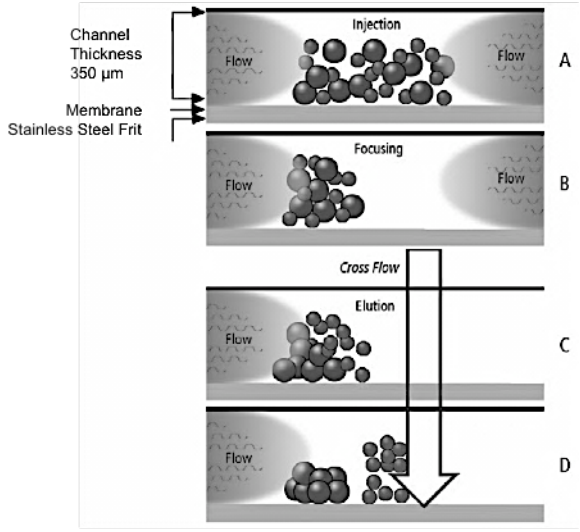

Figure 6.2. Cross section of an asymmetrical flow field flow fractionation (AF4) channel: separation and elution process. http://www.perkinelmer.com

AF4 selectivity is based on the diffusion coefficient of the sample; hence the hydrodynamic radius (R_h) can be determined via the diffusion coefficient from the Stokes–Einstein equation.

$$R_h \equiv \frac{k_B T}{6\pi\eta D_T} \qquad 6.1$$

where D_T represents the translational diffusion coefficient, k_B Boltzman's constant, T the temperature, and η the viscosity of the solvent.
The retention ratio in the normal mode (small particles are eluted first) with constant crossflow according to the AF4 theory is: [9]

$$R = \frac{t_0}{t_{ri}} \approx \frac{6D_i V_0}{F_c w^2} \qquad 6.2$$

where t_0 represents the void time (the time needed for the carrier solvent to pass from inlet to outlet in the channel), t_{ri} elution time, D_i diffusion coefficient of the i_{st} exponent slice, F_c is the crossflow rate, w the channel thickness, and V_0 the void volume (the geometric volume of the channel).
For samples with high size polydispersity, like the enzymatically synthesized amylopectin analogs studied here, a good fractionation of the whole sample can be achieved using a crossflow gradient instead of a constant crossflow. For an exponential regression of a crossflow t_{ri} can be expressed as: [4]

$$t_{ri} = t_{start} + (t_{end} - t_{start}) \left\{ 1 - \exp \left[- \frac{\frac{t_0 w^2 F_{c_{start}}}{6DV_0} - t_{start}}{t_{end} - t_{start}} \right] \right\} \qquad 6.3$$

where t_{start} is the time corresponding to the start and t_{end} the time corresponding to the end of the gradient.
Size distributions can be obtained by determining the relationship between D_T and t_{ri} using established equations for methods with constant crossflow, [10] and their modifications for gradient crossflow. [11, 12] If possible, R_h can be determined online by means of quasi-elastic light scattering (QELS), providing experimental size distributions.
In this study we concentrate on the previously analyzed - with SEC - amylopectin like well-defined branched polysaccharides (Chapter 4), in order to obtain and compare the different distributions and the structural characteristics as well as the two characterization techniques, SEC and AF4. The system used for the separation and characterization of amylopectin analogs was AF4-MALLS-QELS setup. By comparing, fulfilling, correcting and combining the results from two powerful techniques such as SEC and AF4 with multi detection, we were able to show how important the establishment of improved characterization protocols for branched polysaccharides is.

6.2 Experimental

6.2.1 Materials and methods

All chemicals used for the synthesis and AF4 (glucose-1-phosphate (G-1-P), tris(hydroxymethyl)aminomethane (Tris), dithiothreitol (DTT), adenosine monophosphate (AMP), phosphorylase *b*, sulfuric acid, NaN_3, Orcinol) were purchased from Sigma-Aldrich and used without further purification. Glycogen branching enzyme from *Deinococcus geothermalis* (Dg GBE) was kindly provided by R.J. Leemhuis and L. Dijkhuizen, whereas maltoheptaose (G-7) was synthesized as explained in Chapter 2. [13] The water used for analysis and sample preparation was produced by a RiOsTM and Synergy purification system (Millipore, Bedford, MA, USA).

UV-Spectroscopy
^{1}HNMR Spectroscopy
Determination of the degree of polymerization
Determination of the degree of branching
are explained in detail in Chapter 2.

Synthesis of well-defined branched polysaccharides [14]
is explained in detail in Chapter 4.

6.2.2 AF4 analysis

The AF4 equipment, including the asymmetrical channel, Control-Box V3, Flow box P2.1, and the valve box, was obtained from Consenxus (Ober-Hilbersheim, Germany). The channel geometry was trapezoidal with a tip-to-tip length of 286 mm and breadths at the inlet and outlet of 21.2 and 4.7 mm, respectively.
A 350 µm polyester spacer and a pure cellulose membrane with a cutoff point of 10 000 Da from Celgard LLB (Charlotte, NC) were used. During all AF4 experiments, the sample was introduced into the channel using a 100 µL loop injector (Valco Instruments Co., Inc., Houston, TX). A Dawn® Heleos MALLS system fitted with a K5 flow cell and a GaAs laser (λ = 658 nm) from Wyatt Technology Corporation (Santa Barbara, CA, USA) and an RID-10A refractometer from Shimadzu (Kyoto, Japan) were

used as detectors. Online QELS measurements were performed at 142.5° for a time interval of 7 s using a WyattQELS® system (Wyatt Technology Corporation). Prior to use, the carrier (Millipore water containing 0.2 g L^{-1} sodium azide) was carefully degassed and filtered through Durapore GV (0.22 μm*)* membranes (Millipore). The carrier was eluted initially at a flow rate of 1 mL min^{-1} for channel flow in (F_{in}). The crossflow (F_c) was then set at 1 mL min^{-1}, and the channel flow rate (F_{out}) was set at 0.2 mL min^{-1} for the sample introduction and relaxation/focusing period. The sample was injected at 0.1 mL min^{-1} for 600 s. After the injection pump was stopped, the sample was allowed to relax and focus for 60 s.

For elution, F_{out} was set at 1 mL min^{-1}, and F_c was reduced from 1.3 to 0.1 mL min^{-1} for 600 s exponentially, after which it was maintained at 0.1 mL min^{-1} for 900 s. In order to elute the whole sample in case some aggregates existed, the flow was reduced to 0 mL min^{-1} and maintained there for 300 s.

Prior to analysis, the samples were solubilized for 3h at 50℃ and overnight at room temperature in a water bath. All samples were filtered through 0.45 μm filters after dissolving. Sample recoveries were calculated from the ratio of the mass eluted from the channel (integration of the differential refractometric index (DRI) signal) and the injected mass. The injected masses were determined using the sulfuric acid-orcinol colorimetric method (see below).

Data Processing

$\overline{M}_n = \frac{\sum c_i}{\sum \frac{c_i}{M_i}}$, $\overline{M}_w = \frac{\sum c_i M_i}{\sum c_i}$, the polydispersity index $\frac{\overline{M}_w}{\overline{M}_n}$, and $\overline{R}^2{}_g = \frac{\sum c_i M_i R_{gi}^2}{\sum c_i M_i}$ were established using ASTRA software from WTC (version astra 6.1.1 for Windows). The quantities c_i, M_i, R_{gi} were obtained after processing the light scattering (LS) and DRI profiles. The refractive index increment dn/dc used for all the calculations was 0.146 mL g^{-1}. The normalization of photodiodes was accomplished with a low molar mass pullulan standard (P20). Berry extrapolation of the light-scattering equation (Equation 6.4) for the scattered light to an angle of zero was used to obtain M_i and R_{gi}.

$$\sqrt{\left(\frac{Kc}{R_\theta}\right)_i} = \sqrt{\frac{1}{M_i}\left(1 + \frac{16\pi^2 n^2}{3\lambda^2} R_{gi}^2 \sin^2(\theta/2)\right)} \qquad 6.4$$

where K is the optical constant, R_θ is the excess Rayleigh ratio of the solute, λ is the wavelength of the incident laser beam, and θ is the angle of observation. The Berry extrapolation method was used rather than the classic Zimm plot since it allows a more accurate extrapolation for a very large polymer size. [15] Only the seven lower angles (from 29° to 90°) were used for extrapolation. The hydrodynamic radius of the slice i R_{hi} was calculated using D_T from online QELS measurements with the Stokes-Einstein equation (see Equation 6.1) as previously defined. [16, 20]

6.2.3 Sulfuric acid-orcinol colorimetric method.

For the preparation of the sulfuric-orcinol reagent, firstly 1400 mL of concentrated sulfuric acid (98%, d=1.83) was diluted with 650 mL of Millipore water in an ice bath. 2 g of orcinol dissolved in 50 mL of water was added to the diluted sulfuric acid. The sulfuric-orcinol reagent must be kept in the dark, and not used longer than two weeks.
For every sample the glucose content was determined before and after filtration prior to AF4 analysis, in order to determine whether the polysaccharides were properly dissolved. The glucose concentration was determined using a calibration in the range of 0 and 100 µg mL^{-1} established with the solutions of glucose standards with different concentrations using sulfuric-orcinol colorimetric method. Twenty samples per one calibration should be the maximum number of samples analyzed. Degradation of polysaccharides to glucose and spectroscopic colorimetric measurements was done on a continuous flow system from Bran et Luebbe (Plaisir, France) − fluorescent sensitizer, using the sulfuric-orcinol reagent. [17] Samples are injected into this system via an auto-sampler and a pump was used to make intentional air bubbles every second in the analytical line, the samples were heated up to 98 °C and mixed with the reagent. At the end of the line a colorimeter measures the absorbance at 420 nm.

6.3 Results and discussion

All analyzed samples were divided into the same two groups as outlined in Chapter 4. To recall, the first group consisted of polymers synthesized at different reaction times whereas the second group consisted of polymers synthesized with different ratios between the monomer and the primer (different monomer concentrations).

6.3.1 Different reaction time – different degree of branching

The enzymatically synthesized amylopectin analogs that had the same starting ratio of monomer (G-1-P) and primer (G-7), but different reaction times (different degree of branching) during the synthesis were tested using AF4 with DRI, MALLS and QELS. The average degree of branching increased with increase of reaction time as previously shown by ^{1}H-NMR. [18] The properties of the tested samples are shown in Table 6.1, containing the average degree of branching (DB), [19] and the weight-average and number average molar masses ($\overline{M}_w, \overline{M}_n$), determined by AF4/DRI/MALLS, for each sample; the structural parameter ν_h that was determined from R_h, calculated using online QELS measurements, as explained further on in this chapter.

Table 6.1. Properties of the enzymatically synthesized branched polysaccharides with different reaction times (different degree of branching)

S (Time) h	$\frac{G-1-P}{G-7}$	DB [a], %	$\bar{M}_n$, g mol^{-1} AF4 water	$\bar{M}_w$, g mol^{-1} AF4 water	ν_h [b]
1.4 (1)	300	3	1.36 x10^5	5.04x10^5	ND
2.4 (3)	300	7	2.08 x10^5	1.03x10^6	0.27
3.4 (6)	300	7	2.57 x10^5	2.20x10^6	0.33
4.4 (9)	300	8	3.91 x10^5	3.35x10^6	0.30
5.4 (24)	300	10	5.66 x10^5	4.56x10^6	0.32
6.4 (72)	300	12	7.18 x10^5	6.33x10^6	0.32

[a] Determined via ^{1}H-NMR spectroscopy.
[b] slope of the loglog plot of R_h versus molar mass

AF4 analyses of all analyzed polysaccharides from this group showed elution recoveries higher than 97 %, which indicates that the fractionation response was quantitative for all samples.
The $\bar{M}_n$ obtained with AF4 was in the same range or slightly higher than the $\bar{M}_n$, measured with SEC in different solvents and monotonically increased with the increase of the reaction time and the average degree of branching. These results confirm the assumption that not all chains uniformly serve as branching donors and acceptors; some linear amylose chains that are created by the action of phosphorylase plainly serve as branch donors and therefore get completely consumed in the end. This results in fewer chains than the used primer sequences (G-7) at the end of the reaction.

(a) (b)

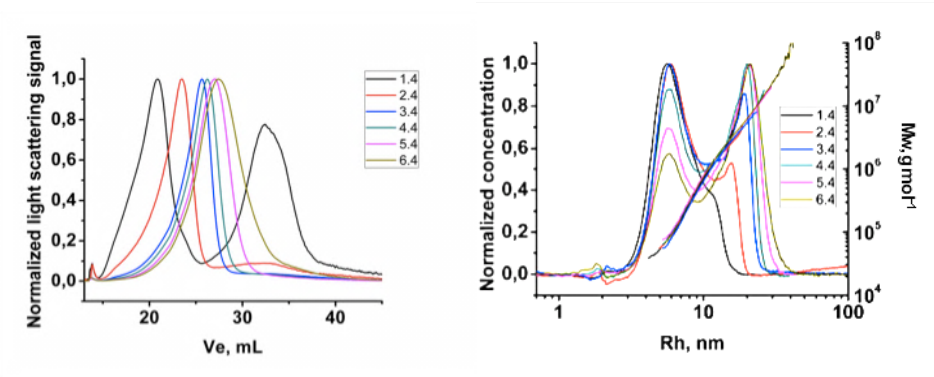

Figure 6.1. (a) Elugrams, **(b)** size, and molar mass distributions of the enzymatically synthesized branched polysaccharides with different reaction times (different degrees of branching).
(sample codes from Table 6.1)

The plots of the LS signal versus V_e for all samples from this group (Figure 6.1 (a)) showed a shift of the peaks to higher V_e with increase of the reaction time, meaning that the longer the reaction time the bigger the molecules are. The sample synthesized for the shortest time, showed an additional LS signal, at a long molecule size, which is most probably an artifact (no concentration for this size).
Size (R_h) distributions measured for each sample, using online QELS, can be seen in Figure 6.1 (b). The R_h was fitted with polynomial fit (fit based on all measured samples) for the improvement of the values for samples measured with lower quality. With increase of the reaction time the R_h values shifted to higher values, indicating an increase in size. The distributions were bimodal for all samples. These results fully agree with the different size distributions observed via SEC measurements. The first population existed in the low R_h area up to 10 nm, whereas the second population existed in the high R_h area with R_h up to 40 nm. Figure 6.1 (b) clearly shows that the amount of low R_h molecules decreases with increase of the reaction time, whereas the amount of molecules with high R_h increases. In both areas, the most dominant R_h shifted towards higher values with increase of reaction time.
The structural information can be obtained from the exponents ν_h or ν_g (hydrodynamic coefficients) using the power-law equations: $\overline{R}_h = K_h \overline{M}_w^{\nu_h}$

and $\overline{R}_g = K_g \overline{M}_w^{\nu_g}$, respectively, in which K_h and K_g represents the corresponding coefficients. The exponent ν should remain the same in the context of these laws. [20] The values of ν_h and ν_g depend upon temperature, polymer–solvent interactions and polymer shape, and: $\nu_h = \nu_g = 0.33$, for a sphere, $\nu_h = \nu_g = 0.5–0.6$ for a linear random coil, [21] and $\nu_h = \nu_g = 1$ for a rod. The corresponding coefficient is not the same and it is dependent on the chosen solvent and the detailed monomer structure. [22]

For the majority of the polysaccharides, for which the R_h values were measurable, structural information was determined from the exponent ν_h (slope of the log-log plot of the hydrodynamic radius versus molar mass) using the power-law equation: $R_{hi} = K_h M_i^{\nu_h}$, and are listed in Table 6.1. Figure 6.2 (a) compiles the experimental data for R_h of all samples from this group, and it is clear that a quality fit can be achieved using the power law equation for R_h > 10 nm. The parameter ν_h varied between 0.27 and 0.33, which is characteristic for compact spheres. [23] This is as expected, since we have hyper(branched) amylopectin analogs very similar to glycogens. [20]

Unfortunately, due to many different molecular sizes in one measured polysaccharide, and a number of the low molecular sizes in each of them, determination of R_g was not adequate for the whole distribution therefore ν_g ($R_{gi} = K_g M_i^{\nu_g}$ slope of the log-log plot of the radius of gyration versus molar mass) values were not good enough due to an inadequate fit.

The apparent molecular density distribution $d_{H_{appLSi}} = \frac{M_{wi}}{\frac{4\pi}{3} R_{hi}{}^3}$ calculated with the fitted R_h can be seen in Figure 6.2 (b). For every sample, for the whole R_h range the density was very high, confirming the highly dense branched structure of the polysaccharides. As expected in the low R_h area we can see a slight increase in the density for the majority of the samples, hence an increase in the branching, whereas in the high R_h area the density becomes the same after a specific reaction time, which means that even though the average degree of branching increases the molecules stop to be further branched in this area after a certain time. Sample 1.4 should have the lowest density of all samples since it was synthesized for only one hour. The observed higher from

expected density is due to the fitting, since the experimental data of this sample was not very good due to small size of the molecules.

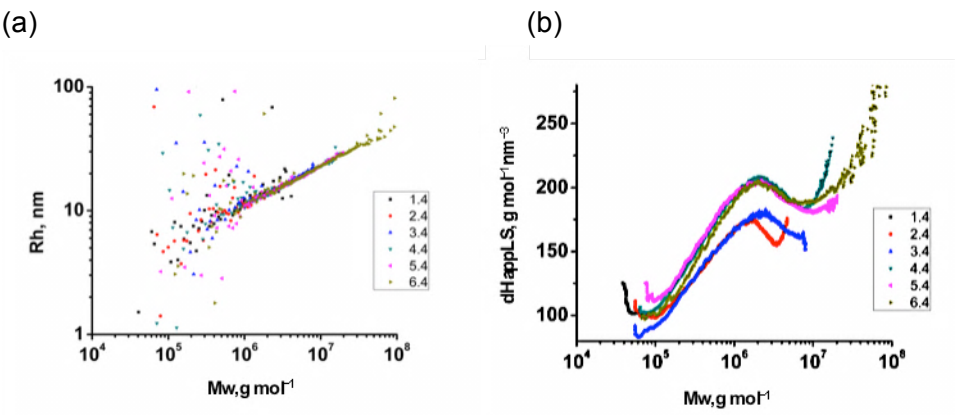

Figure 6.2. (a) R_h (from online QELS measurements) versus molar mass **(b)** the apparent molecular density (dHappLS) versus molar mass.

6.3.2 Different monomer concentration – different degree of polymerization

The amylopectin analogs that had the same reaction time (72h) but a different starting $\frac{G-1-P}{G-7}$ ratio (different degree of polymerization) during the synthesis, were analyzed in the same way as outlined above. From SEC analysis a bimodal distribution of highly branched molecules with a decrease in the average molar mass and size with increase of the monomer concentration can be expected. The properties measured via AF4 are shown in Table 6.2. Both $\overline{M}_n$ and $\overline{M}_w$ decreased constantly with increase of the monomer concentration. The only exception is the sample in which the highest monomer concentration was used (sample 6.7; $\frac{G-1-P}{G-7}$ ratio = 600) − the molar mass is slightly higher than for the $\frac{G-1-P}{G-7}$ ratio = 500. The decrease in the molar mass confirmed that due to increase of the concentration and therefore hindered diffusion some cleaved oligosaccharides did not reach the final destination (other polysaccharide chains) and became branches, but served as new primers for new linear chains. The slight increase in the molar mass of the sample in which the highest monomer concentration was used could be an artifact, however it is more likely an indication for the change of the

enzymatic polymerization mechanism as already observed by SEC. This means that no more oligosaccharides are cleaved, and that the existing branched polysaccharides are simply serving as primers and are being enlarged by G-1-P with phosphorylase as catalyst.

Table 6.2. Properties of the enzymatically synthesized branched polysaccharides with different monomer concentrations (different degree of polymerization)

S	$\frac{G-1-P}{G-7}$	$DB^{a)}$, %	$\bar{M}_n$, g mol^{-1} AF4 water	$\bar{M}_w$, g mol^{-1} AF4 water	ν_h
6.1	50	12	5.52x10^6	4.62x10^7	0.25
6.2	100	12	4.34x10^6	3.64x10^7	0.25
6.3	200	12	2.84x10^6	2.18x10^7	0.28
6.4	300	12	7.18x10^5	6.33x10^6	0.32
6.5	400	11	4.22x10^5	6.02x10^6	0.32
6.6	500	10	1.82x10^5	1.31x10^6	0.33
6.7	600	10	2.06x10^5	2.48x10^6	0.32

a) Determined via ^{1}H-NMR spectroscopy.

Analyzed polysaccharides from this group with a ratio of monomer to primer higher than 100, showed elution recoveries higher than 95 %, which indicates that the fractionation response was quantitative for those samples.

The plots of LS signal versus V_e for all samples from this group (Figure 6.3 (a)) showed a shift of the peak to lower V_e with increase of monomer concentration, meaning that the higher the used monomer concentration the lower the molecular size is.

Size distributions made for each sample, using the online measured R_h (QELS), fitted with a polynomial fit (fit based on all measured samples) can be seen in Figure 6.3 (b). With increase of the monomer concentration R_h values were shifted to lower values in the high R_h area, whereas in the low R_h area the R_h values stayed the same. The distributions were bimodal for all samples. These results fully agree with the different size distributions observed with SEC measurements. The first population existed in the low R_h area up to 10 nm, whereas the second population existed in the high R_h area with R_h up to 80 nm. Figure 6.3 (b) clearly showed that the amount of the polysaccharides in

the low R_h area increased with increase of the monomer concentration, whereas the amount of polysaccharides in a high R_h area increased till the critical monomer concentration ($\frac{G-1-P}{G-7}$ ratio = 500) after which their concentration decreased.

(a) (b)

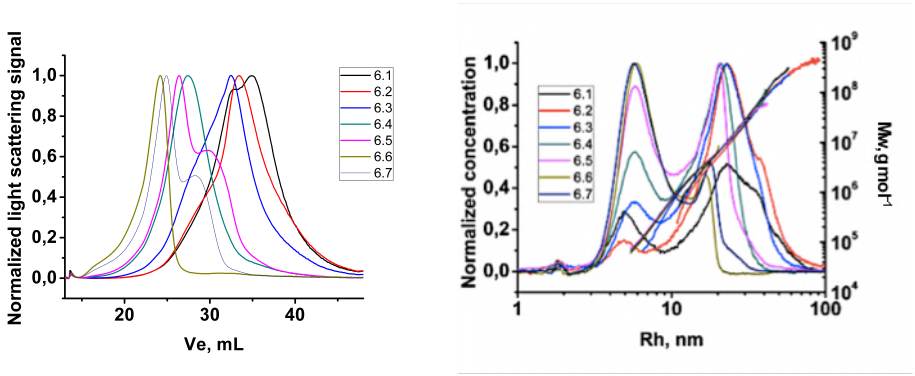

Figure 6.3. (a) Elugrams, **(b)** size, and molar mass distributions of the enzymatically synthesized branched polysaccharides with different monomer concentration (different degree of polymerization).
(sample codes from Table 6.2)

For all polysaccharides, structural information was determined from the exponent ν_h as explained above, and can be seen in Table 6.2. Figure 6.4 (a) shows all experimental data for R_h of all the samples from this group, and it is clear that a quality fit can be achieved. The parameter ν_h varied between 0.25 and 0.33, which is characteristic for compact spheres. For R_g and ν_g values the same problems as discussed above occurred for the majority of samples.

The apparent molecular density (distribution calculated with fitted R_h Figure 6.4 (b)), was very high for all the samples throughout the whole distribution, as expected. For the samples with a lower ratio ($\frac{G-1-P}{G-7}$ ratio < 300) we see an unexpected increase of the density with increase of the monomer concentration, in the low R_h area. Whereas a slight decrease in the density for the same molar mass of the samples synthesized with $\frac{G-1-P}{G-7}$ ratio = 300 and higher is visible, hence increase till the 300 ratio followed by the decrease in the branching of the molecules afterwards. The result for the branching behavior in this area was unexpected, since

SEC analysis showed constant decrease in molar mass for the same size, indicating constant decrease in branching. The difference between SEC results and AF4 results in this area for the lower ratio could be due to the use of universal calibration for SEC analysis and the use of online QELS for AF4 analysis. Additionally, the concentration of these molecules is very low; hence the correct detection can always be questioned. In the high R_h area we can clearly see the confirmation of the SEC results. [14] The decrease of the apparent molecular density is clear, and after a critical monomer concentration an increase is visible, which indicates the change of the mechanism of the branching enzyme, as previously assumed. In Chapter 4 (combination of SEC and MALDI analyses) we proposed that a critical number of molecules existed ($\frac{G-1-P}{G-7}$ ratio > 400), after which the process of diffusion becomes hindered and the branching pattern changes. Unfortunately, solely from AF4 analysis of the whole polysaccharide sample it is not possible to confirm the difference in the branching pattern for samples with a critical monomer concentration. Fraction analysis, or partial degradation of the polysaccharides and further analyses could support the speculations about the different branching pattern. Nevertheless, the critical point after which a change starts to occur is obvious, when the branching density starts to increase.

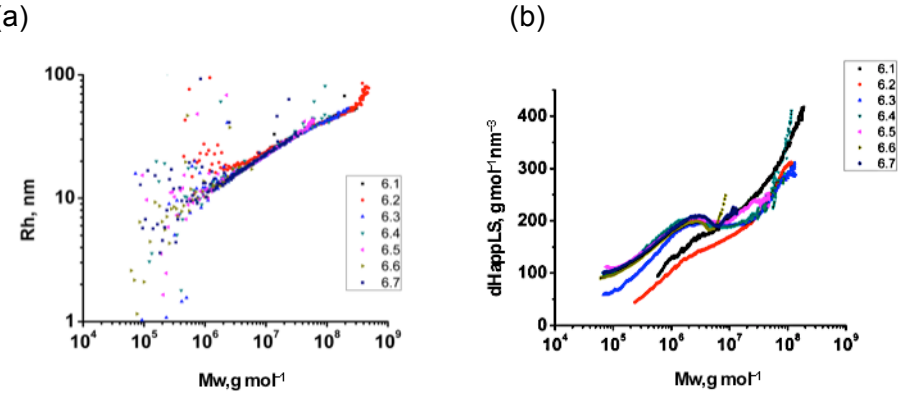

Figure 6.4. (a) The R_h from online QELS measurements versus molar mass **(b)** the apparent molecular density (dHappLS) versus molar mass. (sample codes from Table 6.2)

Recently, very similar branched polysaccharides were synthesized with amylosucrase from *Neisseria polysaccharea* and the branching enzyme (BE) from *Rhodothermus obamensis* using sucrose as a unique substrate, [24] and the effect of the monomer concentration was analyzed with the same AF4 system under similar conditions. [5] It is interesting that the increase of the monomer concentration has a completely opposite effect on the size and structure of the synthesized polysaccharides. In this case, as expected, with increase of the monomer concentration, the molar mass also increased. Whereas, in the low R_h area, the amount of fractions decreased, and in the high R_h area the amount of fractions increased with increase of the monomer concentration. ν_h values were slightly higher but still in the range for a compact sphere. Additionally for some samples, authors were not able to determine the R_g, like in the case of the polysaccharides studied in our work. However, for the majority of the samples they were able to determine R_g, hence ν_g which was the same as ν_h and ρ which was close to the theoretical values of a dense spherical structure. The differences between the two polysaccharide samples are probably due to different enzymes, their mechanisms and substrates used in the polymerizations. Moreover the size distributions were completely different, and the bimodal distribution was more pronounced in our research. Problems with the determination of R_g in our case possibly occur due to variety of branched structures inside one polysaccharide.

6.4 Conclusions

AF4 with multi detection has been confirmed to be a very powerful technique for the analysis of the macromolecular structure of branched polysaccharides. [4, 5, 20] Not only did we successfully determine the size and molar mass distributions of enzymatically synthesized amylopectin analogs, but we also confirmed their highly branched structure.
The assumptions concerning the molecular mechanism of the enzymatic polymerization - used in our work made after SEC (Chapter 4) analysis proved to be correct.
With increase of the reaction time/degree of branching, a constant increase of the molar masses confirmed that some chains serve mostly as branch donors and some as acceptors during the one-pot enzymatic synthesis. Additionally, we clearly showed that in the high R_h area after 6 hours amylopectin analogs stop being branched further on, even though the average degree of branching increases with time branching stops.
With increase of the monomer concentration/degree of polymerization, the observed clear decrease of the molar masses which can be explained by the fact that some of the short oligosaccharides cleaved by Dg GBE serve as primers instead of becoming branches due to hindered diffusion.
Using AF4-MALLS-QELS we clearly determined the macromolecular characteristics of the branched polysaccharides. Concerning structural information based on hydrodynamic radius, we concluded to have highly branched glycogen-like particles in all cases. Unfortunately, due to the low molecular sizes and difficult determination of the radius of gyration, structural information based on the radius could not be taken into account.
In this chapter we showed that AF4 could superbly be used for the fractionation of highly branched polysaccharides with various sizes and degrees of branching. Moreover, we confirmed all the assumptions based on SEC analysis concerning the enzymatic synthesis used in this work.
When SEC and AF4 are compared for the aqueous system, due to the absence of columns (no stationary phase) and no interaction of polysaccharides with the column material, preferably AF4 should be used, especially for bigger molecules. Unsurprisingly, if affordable and

accessible, the combination of these two techniques is the best one for the characterization of branched polysaccharides.

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CHAPTER 7

CONCLUSIONS

The main goals of this thesis were to synthesize a variety of highly defined branched polysaccharides by an enzymatic tandem polymerization using phosphorylase and glycogen branching enzyme, to characterize them and to establish improved characterization techniques for branched polysaccharides. The most important conclusions of this thesis are outlined in this chapter.

General conclusion

In this thesis we presented both the synthesis of amylopectin analogs and their characterization. In Chapter 2 the previously utilized synthesis of amylopectin analogs with potato phosphorylase and glycogen branching enzyme was adjusted with the slight modification of the isolation process of the phosphorylase. Additionally, two different phosphorylases were estimated for the enzymatic synthesis of amylopectin analogs, potato phosphorylase and phosphorylase *b* from rabbit muscle. The best enzymatic synthetic pathway was chosen and used further on in our work. In Chapter 3 several methods for tuning the degree of branching were found and the best one - the reaction time regulation was chosen and combined with the previously established method for tuning the degree of polymerization. The average degree of polymerization of the analogs was calculated from a quantitative spectroscopic determination of the released inorganic phosphate while the degree of branching of the synthesized polymers was determined by ^{1}H-NMR. In order to acquire data that is sensitive to the structure of polymers we used size separation techniques ((SEC (Chapter 4) and AF4 (Chapter 6)) with multi detection). Physical properties of the analogs were determined with DLS, different microscopy, DSC and rheological measurements (Chapter 5).
SEC analysis provided valuable information concerning different distributions, and in combination with MALDI-ToF MS analysis of debranched analogs we unraveled parts of the synthetic mechanism. The AF4 analysis fully supported conclusions made by SEC, and gave some additional information in regard to the structure.
This characterization approach (development of materials; developed materials analyzed with size separation techniques with multiple detections) can help understand synthesis-structure-property relationship of important industrial branched polysaccharides.

Chapter 2

Potato phosphorylase was isolated via a standard and a modified method (using protease inhibitor – PMSF in the modified one). Both phosphorylases were used for the synthesis of branched polysaccharides, and the polymerizations were monitored.

- After comparing their catalytic activity and analyzing the kinetics of the polymerizations thoroughly, we concluded that the modified suspension of potato phosphorylase has a higher activity and a longer lifetime.

Additionally the effect of a reducing agent – DTT on phosphorylase was analyzed.

- DTT in this case, has no effect on the potato phosphorylase.

Phosphorylase *b* from rabbit muscle is commercially available and is genetically more than 75% equal to potato phosphorylase. Therefore we have performed a parallel study of those phosphorylases for the synthesis of amylopectin analogs.

- Phosphorylase *b* has affinity towards branched substrates, hence is more appropriate for the synthesis of amylopectin analogs.
- Lower activity of phosphorylase *b* in comparison to potato phosphorylase is needed for the synthesis of amylopectin analogs.
- A higher degree of branching is achieved with phosphorylase *b*.
- Phosphorylase *b* and Dg GBE resulted in pure, water-soluble amylopectin analog.

For all these reasons, we decided to use phosphorylase *b* for our further research.

Chapter 3

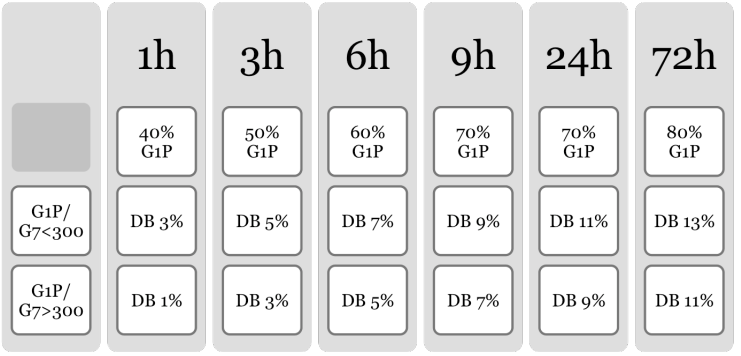
Via an enzymatic tandem polymerization using phosphorylase *b* and Dg GBE various amylopectin analogs with tunable degree of branching and polymerization were synthesized. In order to tune the degree of branching we varied different parameters.

- Varying reducing agents and their concentration we were able to slightly tune the degree of branching.
- We showed that the degree of polymerization depends on the reducing agent, however it is not crucial for the polymerization.
- Different pH values vaguely affect the degree of branching whereas almost not the degree of polymerization.
- By varying the reaction time we succeeded in synthesizing amylopectin analogs with various degrees of branching.

As it was known from literature how to tune the degree of polymerization by varying the ratio between monomer and primer (monomer

concentration), and from our research how to tune the degree of branching, we were able to synthesize a large library of amylopectin analogs.

- For instance, with a high amount of glucose-1-phosphate, and a short time, we can expect relatively high degree of polymerization and a very low degree of branching, whereas for a long time, we can expect high degree of polymerization and very high degree of branching, see below.



Chapter 4

Two groups of amylopectin analogs were tested using SEC with multi detection. Subsequently the analogs were debranched enzymatically and the branching distribution was analyzed with MALDI-ToF MS. In a first group of molecules we varied the reaction time, whereas the monomer concentration was constant – this resulted in a different degree of branching. In a second group of molecules we varied the monomer concentration, whereas the reaction time stayed constant – this resulted in a different degree of polymerization.

- With increase of the reaction time an unexpected constant increase of the molecular weights was detected by SEC even after no more monomer was consumed in the reaction.
  - Some chains serve mostly as branch donors and some as acceptors during the one-pot enzymatic synthesis.
- With increase of the monomer concentration an unexpected constant decrease of the molecular weights was detected.
  - Some of the cleaved short oligosaccharides serve as primers instead of becoming branches due to hindered diffusion.

- In both analyzed groups, two size regions were detected – a low and a high hydrodynamic radius area.
- With increase of the reaction time the high hydrodynamic radius area becomes more dominant, the side chains become longer, and molecules stop being branched after 6h. The low hydrodynamic radius area decreases, but molecules become more branched.
- The branching pattern in this group of samples resembles more a polyethylene branching pattern than an amylopectin one, since the side chain length distribution is very broad and long side chains are visible.
- With increase of the monomer concentration, both the low hydrodynamic radius and the high hydrodynamic radius peaks increased. When the ratio became greater than 400 (critical ratio, critical monomer concentration), the low hydrodynamic radius peak continued to increase whereas the high hydrodynamic radius peak decreased. The synthesis of smaller molecules became preferable and at the critical monomer concentration phosphorylase prefers to catalyze the synthesis of linear chains and prolongs the newly created branches on the low-branched molecules.
- The side chain length distribution became less broad and long side chains became less visible after the critical ratio. We suspect that the branching pattern in the high hydrodynamic radius area changes to a typical amylopectin pattern with many short branches.

For the DMSO/LiBr system we used PFG (PSS) columns that are not typically used for the analysis of starches in DMSO and showed their potential in starch analysis.

In this chapter we tested different solvent systems, both organic and aqueous; confirmed our results and solved parts of the molecular mechanism of the enzymatic polymerization of amylopectin analogs.

Chapter 5

In this chapter we showed the effect of the synthesis conditions and the structure of the samples on their physical properties.

- Using DLS, cryo-TEM, AFM, DSC and rheological measurements we confirmed the conclusions made in Chapter 4.
- The agreement of the particle size measured with different techniques was very good.
- Synthetic amylopectin is stable to retrogradation at 4 °C if the main side chain length is not longer than 12 glucose units and the polydispersity of the side chains is narrow.
- Amylopectin analogs in the form of 20% suspensions have fluid-like behavior, indicating the low strength of hydrogen bonding between the chains and the absence of strong aggregation.

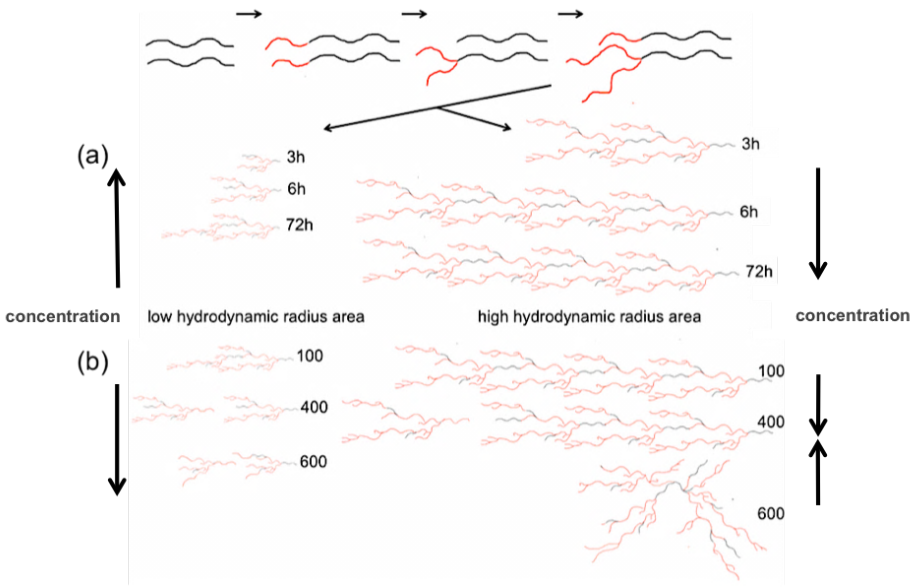
Chapter 6

AF4 with multi detection is a powerful system for the analysis of the macromolecular structure of branched polysaccharides. Therefore we analyzed the same two groups of amylopectin analogs as in Chapter 4 with AF4.

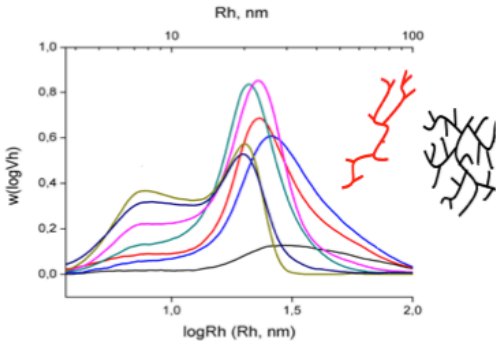
- We determined the size and molecular weight distributions of the analogs; confirmed their highly branched structure and the assumptions concerning the mechanism of synthesis.
- With increase of the reaction time a constant increase of the molecular weights confirmed that some chains serve mostly as branch donors and some as acceptors.
- It is clear that after 6 hours amylopectin analogs stop being branched further in the high hydrodynamic radius area, even though the average degree of branching increases with time.
- With increase of the monomer concentration a clear decrease of the molecular weights is observed and confirms that some of the short oligosaccharides cleaved by Dg GBE serve as primers instead of becoming branches due to hindered diffusion.
- Based on hydrodynamic radius analysis, we concluded that all our analogs have highly branched glycogen-like structure.
- Overall AF4 seems to be a superior characterization technique.

Based on the results obtained by SEC & AF4 characterization in combination with MALDI-ToF MS measurements it was possible to

establish parts of the mechanism of the performed enzymatic tandem polymerization towards synthetic amylopectin analogs, see below.



In general, phosphorylase elongates the used primer (black) and the branching enzyme cleaves short oligosaccharides and transfers them from an α-(1→4) position to an α-(1→6) position. Subsequently phosphorylase elongates both the newly created branches and the elongated primers. By repeating this cycle amylopectin analogs are formed, which is depicted in the upper part of the picture, from left to right. In the synthesized amylopectin analogs two major size distributions (low and high hydrodynamic radius) can be clearly observed – as can be for instance seen in the SEC traces below.



With increase of the reaction time, some polysaccharides created from primer are chopped off into short oligosaccharides by the branching enzyme and serve as further branches even after no more monomer is consumed in the reaction. This is the reason for the observed constant increase in molecular weight over time – see part (a) of the picture above. Additionally this is the reason why the molecular weights calculated based on used primer differ from the molecular weights measured by MALLS – some chains do get consumed and therefore increase the molecular weight of other chains. In the high hydrodynamic radius area after 6h molecules stop being further branched but their concentration increases, whereas in the low hydrodynamic radius area molecules are constantly being branched but their concentration decreases with time. The branching pattern resembles a polyethylene branching patter with both long and short branches (which can be seen in the size distribution picture depicted in red, or in the mechanism for the majority of the samples in the high hydrodynamic area). The branching pattern is established from the branch distribution determined by MALDI-ToF MS, and can be mainly assigned to the high hydrodynamic radius area, due to its higher concentration. Unfortunately, from bulk analysis we cannot be sure about the same/different branching pattern in the two different hydrodynamic radius areas.

With increase of the monomer concentration (the ratio monomer/primer 100, 400, and 600 respectively – see part (b) of the picture above) some oligosaccharides cleaved by glycogen branching enzyme serve as new primers instead of becoming branches (molecules on the left sides of both low and high hydrodynamic radius areas – molecules that begins with a red “primer-branch”), which is possibly due to hindered diffusion. This behavior is the reason for the observed decrease in molecular weight regardless of the constant increase in monomer consumption during the reaction. Additionally this is the reason why the molecular weights calculated based on used primer differ from the molecular weights measured by MALLS. With increase of the ratio, the concentration of molecules in both size regions increases till a critical ratio (400) is reached. The degree of branching is constant in the high hydrodynamic radius area till this critical ratio is reached, whereas the degree of branching slightly decreases in the low hydrodynamic radius area. After this critical ratio, the concentration of the molecules in the high hydrodynamic radius area decreases whereas it continues to

increase in the low hydrodynamic radius area. Additionally molecules become smaller in the high hydrodynamic radius area and their branching pattern changes towards a typical glycogen branching pattern with many short branches and an increased degree of branching (which can be seen in the size distribution picture depicted in black and, in the mechanism for the ratio of 600 in the high hydrodynamic area). After the critical point the degree of branching in the low hydrodynamic radius area either stays the same or slightly decreases.

SUMMARY

Starch (amylose and amylopectin), glycogen and cellulose are natural polymers, and they are all around us. We consume and use such polysaccharides on a daily basis; unfortunately we still do not understand them completely. In order to try to understand as much as possible about the structure and the relationship between molecular structure and the physical properties of these polysaccharides, we synthesized such polysaccharides, characterized and used them as standards for the characterization of natural ones.

Using conventional organic chemistry these polysaccharides are very hard to synthesize. Nevertheless if we mimic nature and use enzymes as catalysts in the synthesis, their production is very easy. Linear amylose is formed by the phosphorylase catalyzed propagation of glucose-1-phosphate while glycogen branching enzyme from *Deinococcus geothermalis* (Dg GBE) introduces branching points at the α-(1→6) position by relocating short oligosaccharide chains. For the characterization of amylopectin analogs we used state-of-the-art techniques such as size exclusion chromatography (SEC), asymmetrical flow field flow fractionation (AF4) with various detectors, dynamic light scattering (DLS), atomic force and cryo transmission electron microscopy (AFM and TEM, respectively).

Chapter 1 describes the most prominent categories of polysaccharides, starch, glycogen and cellulose, as well as their synthetic analogs. Furthermore, it gives an overview of the existing different enzymatic routes to such synthetic polysaccharides, possibilities for their characterization and the characterization of natural carbohydrates.

In Chapter 2 we improved the previously established method for synthesis of branched polysaccharides using potato phosphorylase and Dg GBE, concentrating on improved potato phosphorylase isolation. Better isolation was achieved using protease inhibitor and the advantages in comparison to the standard isolation procedure were outlined. Furthermore a parallel study of potato phosphorylase and phosphorylase *b* from rabbit muscle used in the synthesis of amylopectin analogs was performed and confirmed that phosphorylase *b* is more suitable for the synthesis of branched polysaccharides, because the

products were had a higher purity and as well as a higher average degree of branching.

Chapter 3 outlines the optimization of the *in vitro* tandem polymerization of amylopectin analogs using phosphorylase *b* from rabbit muscle and Dg GBE in order to obtain tunable degrees of branching. The tunable degrees of branching are achieved by changing different reaction conditions such as the pH value, the reducing agent and its concentration and the reaction time. We showed that the best way to obtain different degrees of branching is by regulation of the reaction time. Combining this conclusion with previously established methods for tuning the degree of polymerization by simply varying the monomer concentration, we were able to synthesize a large library of amylopectin analogs.

Since determination of the size distributions of natural polysaccharides is known to be a perplexing task, the analysis of enzymatically synthesized amylopectin analogs (Chapter 3) is more favorable.

Therefore Chapter 4 outlines the analysis of two different groups of well-defined synthetic amylopectin analogs with a tunable degree of branching (2% ÷ 13% determined via ^{1}HNMR) and a tunable degree of polymerization (30 ÷ 350 determined indirectly via UV spectrometry). The systems used for the separation and characterization of these branched polysaccharides was SEC-DMSO/LiBr with a PFG column (non-standard column for polysaccharides) and multi detection (refractive index detector, viscosity detector and multi angle light scattering detector (MALLS)). Additionally we used SEC water and SEC-50mM $NaNO_3$ system with multi detection. To further understand the branching pattern in such polysaccharides, they were debranched enzymatically and the side chain length distribution was investigated by MALDI-ToF MS analysis. Additionally, we were able to propose parts of the molecular mechanism of this enzymatic synthesis.

In order to determine the structural characteristics of the same analogs, DLS, AFM and cryo-TEM were used and the results are described in Chapter 5. Additionally, we analyzed the rheological characteristics of 20% suspensions of polysaccharide analogs, showed their behavior at 4 °C and found the critical value of the side chain length for retrogradation to be 12 glucose units. Moreover, the comparison of the results with SEC analyses showed a complete agreement with the observations made in Chapter 4 concerning the enzymatic synthesis mechanism.

Chapter 6 describes a detailed investigation of the same two groups of synthetic amylopectin analogs previously analyzed in Chapter 4, using AF4 coupled with MALLS with online quasi-elastic light scattering (QELS). We proved that this is a very powerful technique for the determination of the macromolecular structure of branched polysaccharides which overcomes some crucial problems found in conventional SEC analysis.

The overall conclusions and the elucidated mechanism of the enzymatic polymerization throughout the thesis are summarized in Chapter 7.

SAMENVATTING

Zetmeel (amylose en amylopectine), glycogeen en cellulose zijn natuurlijke polymeren die je overal om je heen kunt vinden. Dagelijks consumeren en gebruiken wij zulke polysachariden; echter begrijpen wij ze nog niet helemaal. In een poging zo veel mogelijk te weten te komen over de structuur en de relatie tussen de moleculaire structuur en de fysische eigenschappen van deze polysachariden, hebben we een aantal polysachariden gesynthetiseerd en gekarakteriseerd om deze vervolgens te gebruiken als standaard voor de karakterisatie van natuurlijke polysachariden.

Polysachariden zijn lastig te synthetiseren via traditionele organische chemie. Desalniettemin, wanneer we de natuur imiteren en enzymen als katalysator gebruiken kunnen we deze producten erg eenvoudig synthetiseren. Lineair amylose wordt gevormd door de fosforylase gekatalyseerde propagatie van glucose-1-fosfaat terwijl het glycogeen vertakkingsenzym van *Deinococcus geothermalis* (Dg GBE) vertakkingspunten op de α-(1→6) positie introduceert door verplaatsing van korte oligosachariden ketens. Voor de karakterisering van amylopectine analogen gebruikten wij “state-of-the-art” technieken zoals size exclusion chromatography (SEC), asymmetrical flow field flow fractionation (AF4) met verschillende detectoren; dynamische lichtverstrooiing (DLS), atomic force en cryogene transmissie-elektronenmicroscopie (respectievelijk AFM en TEM).

Hoofdstuk 1 beschrijft de meest prominente catogorie polysachariden, zetmeel, glycogeen en cellulose, alsmede hun synthetische analogen. Daarnaast wordt er een overzicht gegeven van de verschillende bestaande enzymatische routes voor het verkrijgen van synthetische polysachariden, de mogelijkheden voor hun karakterisatie en de karakterisering van natuurlijke koolhydraten.

In Hoofdstuk 2 verbeteren we de eerder ontwikkelde methode voor de synthese van vertakte polysachariden gebruikmakend van aardappel fosforylase en Dg GBE, waarbij de focus ligt op het verbeteren van de isolatie van aardappel fosforylase. Een verbetering in de isolatie was

bereikt door het gebruik van een protease inhibitor en de voordelen vergeleken met de standaard isolatieprocedure worden beschreven. Bovendien werd een parallelle studie uitgevoerd naar aardappel forforylase en fosforylase *b* verkregen uit konijnen spier gebruikt in de synthese van amylopectine analogen. Hierin werd bevestigd dat fosforylase *b* geschikter is voor de synthese van vertakte fosforsachariden, omdat de producten schoner zijn en een hogere gemiddelde graad van vertakking bezitten.
Hoofdstuk 3 beschrijft het optimaliseren van de *in vitro* tandem polymerisatie van amylopectine analogen, gebruikmakend van fosforylase *b* verkregen van konijnen spier en Dg GBE zodat er controle over de graad van vertakking is. De mate waarin de vertakkingsgraad aangepast kan worden, wordt bereikt door verandering van de reactie condities zoals de pH waarde, het reductiemiddel en de concentratie hiervan, aslmede de reactie tijd. We laten zien dat het variëren van de reactie tijd de beste manier is om verschillende gradaties van vertakking te krijgen. Door combinatie van de reactietijd en eerder vastgestelde methoden voor het controleren van de graad van polymerisatie door het variëren van de monomeer concentratie, kon er een grote bibliotheek van amylopectine analogen gesynthetiseerd worden.
Omdat het bekend is dat het bepalen van de deeltjesgrootteverdeling van natuurlijke polysachariden lastig is, gaat de voorkeur uit naar de analyse van enzymatisch gesynthetiseerde amylopectine analogen (hoofdstuk 3).
Daarom wordt in Hoofdstuk 4 de analyse van twee verschillende groepen goed gedefinieerde synthetische amylopectine analogen met een regelbare graad van vertakking (2 ÷ 13% bepaald met ^{1}HNMR) en regelbare graad van polymerisatie (30 ÷ 350 indirect bepaald *via* UV spectrometrie) behandeld. De systemen voor het scheiden en karakteriseren van de vertakte polysachariden waren SEC-DMSO/LiBr met PFG-kolom (geen standaard kolom voor polysachariden) en meervoudige detectie (brekingsindex detector, viscositeit detector en multi angle light scattering detector (MALLS)), SEC water en SEC-50mM $NaNO_3$ met meervoudige detectie. Om verder inzicht in het vertakkingspatroon van dit soort polysachariden te krijgen, werden de vertakkingen enzymatisch verwijderd en de zijketenlengte distributie bestudeerd door middel van MALDI-ToF MS analyse. Op basis van deze resultaten konden wij delen van het moleculaire mechanisme van deze enzymatische synthese verklaren.

Om de structurele kenmerken van deze polysaccharide analogen te bepalen, werden DLS, AFM en cryo-TEM gebruikt en de resultaten hiervan worden beschreven in Hoofdstuk 5. We hebben de reologische karakteristieken van 20% suspensies van polysacharide analogen geanalyseerd en hun gedrag bij 4 °C is onderzocht. Daarnaast vonden we dat de kritische waarde van de zijketenlengte voor retrogradatie 12 glucose eenheden is. Bovendien, bij vergelijking van deze resultaten met de SEC analyse, bleek deze volledig in overeenstemming met de gemaakte observaties in Hoofdstuk 4 met betrekking tot het mechanisme van de enzymatische synthese.
Hoofdstuk 6 beschrijft een gedetailleerd onderzoek naar dezelfde twee synthetische amylopectine analogen zoals eerder beschreven in hoofdstuk 4, waarin AF4 gekoppeld met MALLS met online quasi-elestic light scattering (QELS) wordt gebruikt. Uit dit onderzoel blijkt dat dit een zeer krachtige techniek is voor het bepalen van de macromoleculaire struktuur van vertakte polysachariden, waarbij deze techniek enkele cruciale problemen van conventionele SEC analyses ondervangt.
De uiteindelijke conclusies en het voorgestelde mechanisme van de enzymatische polymerisatie zoals beschreven in dit proefschrift, zijn samengevat in hoofdstuk 7.

LIST OF PUBLICATIONS AND CONFERENCES

Publications

Ciric, J., Oostland, J., de Vries, J. W., Woortman, A. J., & Loos, K. (2012). Size exclusion chromatography with multi detection in combination with matrix-assisted laser desorption ionization-time-of-flight mass spectrometry as a tool for unraveling the mechanism of the enzymatic polymerization of polysaccharides. *Analytical chemistry*, *84*(23), 10463-10470.

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Selected Oral Presentations

Zernike Vlieland Conference, Vlieland, The Netherlands, May 2013

Serbian Chemical Society (invited speaker), Belgrade, Serbia, April 2013

POLYCHAR21, Gwangju, South Korea, March 2013
Jürgen springer prize for a young scientist presentation

ISPAC2012, Kerkrade, The Netherlands, June 2012

63. Starch Convention, Deltmond, Germany, April 2012

Dutch Polymer Days, Lunteren, The Netherlands, March 2012

EPNOI, Wageningen, The Netherlands, August 2011.

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