Polymerization of hyperbrached polysaccharides by combined biocatalysis
van der Vlist, Jeroen

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CHAPTER 2

Synthesis of hyperbranched polysaccharides

SUMMARY

An enzymatic tandem reaction is described in which the enzymes phosphorylase and Deinococcus geothermalis glycogen branching enzyme (GBE_{Dg}) catalyze the synthesis of branched polyglucans from maltoheptaose. Phosphorylase consumes glucose-1-phosphate (G-1-P) and polymerizes linear polyglucans while GBE_{Dg} introduces branch points in situ on $\alpha(1\rightarrow6)$ positions by reshuffling short terminal oligosaccharides. The resulting hyperbranched polyglucans were analyzed via methylation analysis and a chemo enzymatic assay (involving the anthrone assay, BCA assay and the debranching assay with isoamylase). MALDI-ToF and $^1$H-NMR were used to confirm the linkage pattern and the degree of branching.
2.1 INTRODUCTION

Polysaccharides fulfil in nature multiple functions. Cellulose fibres function as structural material in wood, starch is the energy storage molecule of plants and the chitin exoskeleton of insects protects the animal’s body. These polysaccharides are high molecular weight and stereo regular biopolymers. These special properties are difficult to control with conventional polymer chemistry and hence most glycoscience is based on modifying existing polysaccharides rather than synthesizing them. In this chapter, a method is described to synthesize a polysaccharide from scratch. To obtain the above described properties enzymes are used (just like in nature) for the construction (see Figure 1.12).

All starting materials, biocatalysts and products as shown in this chapter can be derived from potatoes, illustrating the versatility of the potato as a renewable resource:

- Phosphorylase, in this research isolated from potato tubers, is the key enzyme of the polymerizations as shown in this chapter.
- The branching enzyme or Q-enzyme was first discovered in potato juice\(^1\) and is responsible for the branched character of the amylopectin component in potato starch.
- In industry, cyclodextrins are enzymatically produced from potato starch. Cyclodextrin glycosyltransferase (CGTase; EC 2.4.1.19) is employed to obtain a mixture of \(\alpha\)-, \(\beta\)-, and \(\gamma\)-cyclodextrins\(^2\). In this research, cyclodextrins are converted to maltoheptaose and used as a primer.
- The monomer used, glucose-1-phosphate (G-1-P and also known as Cori ester), was first found in frogs by Cori and Cori\(^3\), but can also be isolated from potatoes\(^4\) and plays a role in energy metabolism of plant and animal.
- Amylose and amylopectin are the main components of potato tubers. The structures as synthesized in this chapter are their synthetic analogues.

2.1.1 Synthesis of maltoheptaose

Linear \(\alpha(1\rightarrow4)\) linked glucose residues, with a minimal length of three residues, are suitable as a primer site for the phosphorylase catalyzed polymerization. However, the reaction velocity differs significantly if the polymerization is primed with short primers\(^5\)-\(^7\), resulting in a broad molecular weight distribution\(^8\). Therefore, the synthesis of a monodisperse primer is of the utmost importance for the production of materials of uniform length.
The enzymatic and/or acid catalyzed hydrolysis of starch results in a mixture of different lengths of oligosaccharides that are capable of priming the reaction. The purification is, however, difficult. This method is therefore not suitable for our purposes, as broad molecular weight distributions, after enzymatic polymerization, are the most likely result.

However, the acid catalyzed hydrolysis of cyclodextrins results in monodisperse oligosaccharides (see Figure 2.1). Here we use a 7-membered cyclic dextrin, β-cyclodextrin, to obtain a linear primer of 7 glucose residues (maltoheptaose).

**Figure 2.1:** Acid catalyzed hydrolysis of β-cyclodextrin.

A concentrated solution of β-cyclodextrin was heated for 2 hours at reflux temperature in a diluted hydrochloric acid system. The glycosidic bonds were randomly cleaved from which about 10% underwent a single cleavage resulting in maltoheptaose. A minor part had more than a single cleavage resulting in smaller oligosaccharides and about 90% of the β-cyclodextrin is not cleaved at all. Unwanted smaller oligosaccharides were removed by precipitation in cold absolute ethanol. Maltoheptaose precipitates while the shorter oligosaccharides dissolve in small concentrations. The unreacted β-cyclodextrin can be removed by complexing the β-cyclodextrin with p-xylene. P-xylene fits in the hydrophobic interior of the cyclodextrin and forms an inclusion complex that is insoluble in water. The precipitated β-cyclodextrin complex can be re-used after thoroughly rinsing with water. The complete removal of β-cyclodextrin is of the utmost importance since it inhibits the phosphorylase enzyme in subsequent reactions. The purity of the primer was checked with 1H-NMR and MALDI-ToF.
A more efficient way to produce a monodisperse primer out of cyclodextrins is with the use of the enzyme cyclodextrinase. Cyclodextrinase from *K. oxytoca* converts 50% from the cyclic form to the linear form\(^\text{10}\). However, the commercial availability of this enzyme is limited.

### 2.1.2 Isolation of potato phosphorylase

As mentioned before, potato tubers are a rich source of phosphorylase. This, together with the availability and the ease of isolation from the tubers makes the potato the ideal source of phosphorylase.

The isolation of phosphorylase starts with the disintegration of peeled potatoes, first with a kitchen blender and after that with an ultra-turrax blender. Sodium bisulfite was added as an anti-oxidant to prevent blackening of the potato slurry\(^\text{11}\). Phenol oxidases, in high concentrations present just below the skin of the potato, catalyze the oxidation of proteins inducing a blackening of the potato slurry and provoking a reduced enzyme activity\(^\text{12}\). The potato slurry was pressed through a kitchen towel and the solids were discarded. The remaining potato juice was a mixture of proteins, water soluble components and enzymes, including \(\alpha\)-amylase. \(\alpha\)-amylase is able to hydrolyze glycosidic \(\alpha(1\rightarrow4)\) linkages and depolymerizes amylase. Removal of \(\alpha\)-amylase is therefore necessary and can be done with a heat treatment at 55.5 °C. The \(\alpha\)-amylase denaturates at this temperature and can be removed via centrifugation and/or filtration.

To isolate the phosphorylase enzyme from the potato juice, ammonium sulfate precipitation was used. Ammonium sulfate precipitation is the specific use of a salting-out technique in which the ionic strength of the solution is increased by the addition of ammonium sulfate. Enzymes precipitate at different salt concentrations making it possible to isolate phosphorylase. First, an ammonium sulfate concentration was chosen in which all unwanted enzymes precipitate. After removing the precipitate, the phosphorylase was salted-out and re-suspended in a citric acid buffer.

This enzyme suspension is already suitable for enzyme catalyzed polymerizations. Further purification results in a more pure phosphorylase suspension. Dialysis was in this research used to remove ammonium sulfate and an ultra filtration membrane was used to remove all components with molecular weights smaller than 100 kDa and to concentrate the solution.
SYNTHESIS OF HYPERBRANCHED POLYSACCHARIDES

SPECTROSCOPIC PHOSPHATE DETERMINATION
The phosphorylase catalyzed reaction can be followed by UV-spectroscopy. Since this reaction yields one inorganic phosphate (P,) per cycle, a quantitatively spectroscopic determination of the [P,] gives information about the amount of consumed G-1-P. Therefore, the molecular weight of the amylose chains produced can be calculated at any time of the reaction.

Fiske and Subbarow\textsuperscript{13} developed a spectroscopic method to measure the [P,] in blood and urine. The method is based on the formation of a blue phosphate molybdate complex that is reduced by a reducing agent to form phosphomolybdic acid. Phosphomolybdic acid is much more readily reduced to blue molybdous compounds than molybdic acid itself. However, the rather low pH that is used makes this method not directly suitable for the measurement of [P,] in systems with labile phosphate esters like G-1-P as the esters will hydrolyze during the measurement\textsuperscript{14}. With the addition of acetate buffer, the pH can be increased to pH 4.2. Under these conditions, the labile G-1-P is much more stable and will not contribute to the inorganic phosphate measurement.

2.1.3 Structural analysis of branched \( \alpha \)-glucans
In order to resolve the linkage pattern of the branched \( \alpha \)-glucans both chemical and enzyme based techniques are used. These techniques together with \( ^1\text{H-NMR} \) and MALDI-ToF provide information about the linkage pattern, average branch length, branch length distribution, number of non-reducing groups and the average degree of branching.

METHYLATION ANALYSIS
Methylation is a technique in which the free hydroxy groups of the polysaccharide are fully methylated. The methylated polysaccharides are subsequently cleaved by an acid catalyzed hydrolysis into the corresponding methylated monosaccharides. In the next two steps, the C1 hydroxy group is reduced to prevent ring closure and the remaining hydroxy groups are acetylated. The acetyl groups mark the original linkage position of the polysaccharide and give information about the linkage pattern (see \textsc{figure} 2.2). The resulting partially methylated alditol acetates (PMAA’s) were analyzed by GC-FID analysis and identified by retention time.
FIGURE 2.2: Fragment of a branched polysaccharide and the corresponding partially methylated alditol acetates. The acetyl groups mark the original linking position while the methyl groups mark the originally free hydroxy groups.

STRUCTURAL ANALYSIS VIA CHEMO ENZYMATIC WAYS

With a combination of methods, which rely on enzymatic or chemical assays, different glucose residues that are connected differently can be identified in a polysaccharide in order to elucidate the linking pattern and structure (see TABLE 2.1 and FIGURE 2.3).

TABLE 2.1: Enzymatic and chemical assays used for the structural analysis of polysaccharides.

<table>
<thead>
<tr>
<th>Groups</th>
<th>abbreviation</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reducing groups</td>
<td>[red. groups]</td>
<td>2,2′-bicinchoninic acid (BCA)</td>
</tr>
<tr>
<td>Non-reducing end groups</td>
<td>[non. red. end groups]</td>
<td>Rapid Smith degradation (RSD)</td>
</tr>
<tr>
<td>Glucose residues</td>
<td>[glc. residues]</td>
<td>Anthrone</td>
</tr>
</tbody>
</table>

The degree of branching can be calculated when both the amount of non-reducing end groups (non. red. end groups) and reducing groups (red. groups) is known, together with the total amount of glucose residues (see EQUATION 2.1).
SYNTHESIS OF HYPERBRANCHED POLYSACCHARIDES

degree of branching (%) = \[ \frac{[\text{non.red. end groups}] - [\text{red. groups}]}{[\text{glc. residues}]} \] \quad 2.1

With the Rapid Smith Degradation (RSD; oxidation with periodate, reduction with sodium borohydride and hydrolysis with sulphuric acid), the number of non-reducing end groups can be determined. The RSD assay produces glycerol from the non-reducing terminal groups of α-glucans and erythritol from the remaining glucose residues. Subsequently, the concentration of glycerol can be measured with a glycerol kinase assay\textsuperscript{15}. It is important to prepare G-1-P free samples for the RSD assay as residual G-1-P is also converted to glycerol.

FIGURE 2.3: BCA assay, RDD assay and anthrone assay.

The amount of reducing groups can be assayed via the BCA method. In this assay, a copper ion (Cu\textsuperscript{2+}) is reduced by the reducing end of the carbohydrate. The reduced copper ion (Cu\textsuperscript{+}) forms a deep-blue complex with BCA and can be quantified with UV-VIS measurements. If the BCA assay is used before ([A]) and after ([A']) the specific hydrolysis of the α(1→6) glycosidic linkages, the amount of side chains can be calculated. α(1→6) Glycosidic linkages can be hydrolyzed by the enzyme isoamylase or pullulanase.

After the specific hydrolysis of branched polysaccharides, more reducing groups become available and hence colour development is more pronounced. When the BCA assay is performed before and after the specific hydrolysis of the α(1→6) linkages, the degree of branching can even be calculated without performing the RSD assay (see EQUATION 2.2).

The amount of glucose residues can be determined with the anthrone assay. The assay is based on the dehydration of glucose residues to furfural derivatives, e.g. hydroxymethylfurfural. Furfural derivatives react with anthrone to form a green-blue colour that can be quantified with UV-VIS measurements.
DETERMINATION OF THE DEGREE OF BRANCHING WITH $^1$H-NMR

The area below signals in a $^1$H-NMR spectrum are proportional to the amount of protons responsible for the signal, and can be used to obtain quantitative information about the structure of the material. The ratio of the $\alpha/\beta$ anomers, the degree of polymerization, degree of branching and the average branch length of polysaccharides can be quantitatively determined$^{16}$. Only good resolved and isolated signals with a high signal to noise ratio give accurate information and can be integrated.

**Table 2.2**: Chemical shift of the anomic protons of maltoheptaose and the position of the $H1(1\rightarrow4,6)$ linkage of a branched glucan ($^1$H-NMR, 300 MHz, $D_2O$).

<table>
<thead>
<tr>
<th>Group</th>
<th>Chemical shift (ppm)$^{17}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1(r)(\alpha)</td>
<td>5.23</td>
</tr>
<tr>
<td>H1(r)(\beta)</td>
<td>4.65</td>
</tr>
<tr>
<td>H1(m)</td>
<td>5.38</td>
</tr>
<tr>
<td>H1(n)</td>
<td>5.33</td>
</tr>
<tr>
<td>H1(1\rightarrow4,6)</td>
<td>5.00</td>
</tr>
</tbody>
</table>

The anomic protons of an $\alpha$-D-glucose residue appear as isolated and good resolved signals between 4.0 and 5.5 ppm and meet the above described criteria (see Figure 2.4). The other protons of an $\alpha$-D-glucose residue appear in the range of 3.2 to 3.9 ppm as a complex series of overlapping signals. In the case of an amylase chain or a branched amylase chain, anomic protons are present in the non-reducing end group (H1(n)), the reducing group (H1(r)), glucose residues in the middle of a chain (H1(m)) and glucose residues at intersection points (H1(1\rightarrow4,6)). All anomic protons give signals at different chemical shifts due to small differences in chemical environment. Table 2.2 gives an overview of the chemical shift (expressed in ppm) of the different anomic protons of a branched amylase.

The anomic proton of $\alpha(1\rightarrow4,6)$ linked sugar residues can be used to determine the amount of branch points while the anomic proton of internal linked $\alpha$-D-
Synthesis of hyperbranched polysaccharides

Glucose residues together with the non-reducing anomeric proton can be used to determine the total amount of glucose residues. The ratio of the surfaces below the signals gives the degree of branching, as follows from Equation 2.3.

\[
\text{Degree of branching} = \frac{\text{H}1(1,4 \rightarrow 6)}{\text{H}1(m) + \text{H}1(n) + \text{H}1(1,4 \rightarrow 6)} \times 100\% \quad \text{(2.3)}
\]

To enhance the \(^1\text{H}\)-spectrum (see Figure 2.4) several precautions were taken:

- Hydroxy protons from the sugar residues were exchanged with deuterium in order to minimize interference in the \(^1\text{H}\)-NMR measurement.
- The probe was tuned for each sample to ensure optimal signal-to-noise ratio (S/N).
- Measurements were done at 50 °C.
- HOD signal was pre-saturated before each measurement.
- The delay between pulses was 10 seconds to ensure complete relaxation. This is more than 5 times the slowest relaxing proton of a sugar residue \((T_1 H4(n) < 2.0 \text{ s}^{18})\).

**Figure 2.4**: \(^1\text{H}\)-NMR spectrum of a branched polysaccharide measured in \(D_2O\) at 50 °C. The HOD signal is visible as a small bump due to the pre-saturation.
2.2 EXPERIMENTAL

2.2.1 Materials and chemicals

β-Cyclodextrin hydrate (Fluka), α-D-glucose-1-phosphate disodium salt hydrate (Sigma), 3-(N-morpholino) propanesulfonic acid (MOPS, Sigma), tri sodium citrate (Merck), ammoniummolybdate (Fluka), potassiumpyrosulfite (Fluka), sodium sulfite (Fluka), metol (Fluka), anthrone (Fluka), 2,2'-bicinchoninic acid (BCA, Fluka), ammonium sulfate (Merck), sodium bisulfite (Acros), p-xylene (Merck) and isoamylase (Aldrich) where used as received. Potatoes were bought at the local grossery. Starch V was a donation of Avebe (Foxhol, Groningen). Glycogen branching enzyme from *Deinococcus geothermalis* (GBEDG) was kindly provided by M. Palomo Reixach, M.J.E.C. van der Maarel and L. Dijkhuizen from the Center for Carbohydrate Bioprocessing.

2.2.2 Analysis and equipment

UV-SPECTROSCOPY

UV-spectroscopy measurements were performed on a PYE Unicam SP8-200 UV/VIS spectrophotometer.

$^1$H-NMR SPECTROSCOPY

$^1$H-NMR spectra were recorded on a Varian VXR spectrometer operating at 300 or 400 MHz at ambient temperatures. Dimethyl-2-silapentane-5-sulfonic acid (DSS) was used as an external reference.

$^3$H-NMR spectra used for the determination of the degree of branching were recorded on a Varian Inova 500 MHz spectrometer at 50 °C with pre-saturation of the HOD resonance. 2,2-Dimethyl-2-silapentane-5-sulfonic acid (DSS) was used as an external reference. Complete relaxation of the protons was ensured by taking a 10 second pause between pulses.

INFRARED SPECTROSCOPY

ATR infrared spectra were recorded on a Bruker IFS88 spectrometer equipped with a MCT-A detector at a resolution of 4 cm$^{-1}$ using an average of 50 scans for sample and reference.
MALDI-TOF

MALDI-ToF MS measurements were performed on a Voyager-DE PRO spectrometer in linear (positive ion) mode with 2,5-dihydroxybenzoic acid (DHB) as a matrix. The matrix solution was made by dissolving DHB (0.2 M) in a 1:1 v/v water/acetonitrile solution. Analyte solution was made by dissolving the product in water R.O. (4 mg·mL⁻¹). Matrix and analyte were mixed in 1:1 v/v ratio. 5 µL of this mixture was deposited on the target and dried in vacuo at 40 °C.

GAS CHROMATOGRAPHY

GC-FID was performed with a RTX 5 Sil MS column (30 m x0.25 mm, Restek) using a temperature program (2 min isothermal at 140 °C, then heated to 260 °C with 8 °C/min)⁹.

ISOLATION AND PURIFICATION OF POTATO PHOSPHORYLASE

2.5 kg potatoes were peeled, cleaned and shredded with a kitchen blender. 100 mL citrate buffer (pH 6.2, 50 mM, 0.02% NaNO₃) and 500 ppm sodiumbisulfite were added to the potato slurry. All of the following preparations were performed while cooling in an ice bath if not otherwise stated. All centrifugation steps were done at 7500 rpm at 4 °C for 20 min. The potato slurry was mixed and disintegrated with an ultra-turrax for 10 min at 7000 rpm and pressed through a kitchen towel. The resulting potato juice was centrifuged to remove the remaining solids. The supernatant was heated to 55.5 °C for 40 min to denature the α-amylase. The potato juice was cooled, ammonium sulfate (100 g·L⁻¹) was added, and the mixture was stirred for 30 min. The potato juice was centrifuged and the solids were discarded. The supernatant was saturated with ammonium sulfate (250 g·L⁻¹) and stirred for 30 min. The precipitated phosphorylase was isolated by means of centrifugation. The phosphorylase was suspended in 200 mL citrate buffer and ammonium sulfate was added (50 g·L⁻¹). This mixture was stirred for 30 min. The phosphorylase suspension was centrifuged and the residue was re-suspended in citrate buffer (pH 6.2, 50 mM, 0.02 % NaNO₃). The phosphorylase suspension was dialyzed against citrate buffer (pH 6.2, 50 mM, 0.02% NaNO₃) and was concentrated with a stirred Amicon cell equipped with a Millipore ultra filtration membrane (100 kDa).
CHAPTER 2

CLONING, EXPRESSION AND PURIFICATION OF THE GLYCOGEN BRANCHING ENZYME

Over expression of GBE<sub>dc</sub> was achieved by overnight growth of <i>E. coli</i> BL21(DE3) Star cells containing the corresponding plasmid at 37 °C and 210 rpm in Luria-Bertani medium supplemented with 50 μg·mL<sup>-1</sup> ampicillin. The cells were harvested by centrifugation (10 min at 10,000 x g). The pellets were re-suspended in 50 mM sodium phosphate buffer, pH 8.0, and the cells were disrupted by sonication (7 times for 15 s at 7 μm with 30 s intervals) and centrifuged (30 min at 15,000 x g). The enzyme was found as a soluble protein in the supernatant and purified by His tag affinity chromatography using a HiTrap chelating column (Amersham Pharmacia, Uppsala, Sweden) charged with nickel sulfate. The enzyme was eluted with a linear gradient of 0 to 500 mM imidazol on an Äkta prime purification system (Amersham Pharmacia). Fractions containing the enzyme were pooled and dialyzed against sodium phosphate buffer (25 mM, pH 8.0).

ACTIVITY ASSAY PHOSPHORYLASE

150.4 mg (400 μmol) glucose-1-phosphate and 4.54 mg (4 μmol) maltoheptaose were dissolved in 3 mL citrate buffer (pH 6.2, 50 mM, 0.02% NaN<sub>3</sub>). The pH was re-adjusted to pH 6.2, filled to 3.9 mL and heated to 37 °C. 100 μL Phosphorylase suspension was added and mixed. Immediately afterwards, a 100 μL aliquot (blanco) was taken and processed as described in the PARAGRAPH SPECTROSCOPIC PHOSPHATE DETERMINATION. After 10 min another 100 μL aliquot was taken and processed as described in PARAGRAPH SPECTROSCOPIC PHOSPHATE DETERMINATION. 1 unit is defined as the amount of phosphorylase suspension that can release 0.1 mg inorganic phosphate (P<sub>i</sub>) per 3 min<sup>20</sup>.

ACTIVITY ASSAY ISOAMYLASE

75 μL sodium acetate buffer (500 mM, pH 3.5), 350 μL 1% starch solution (must be heated to 80 °C for 0.5 hour before use) and 75 μL isoamylase solution were mixed and incubated for 15 minutes at 40 °C. The mixture was cooled to room temperature and 500 μL I<sub>2</sub>/KI solution was added together with 11.5 mL water R.O. The absorption was measured on an UV-VIS spectrophotometer at a wavelength of 610 nm. One unit is defined as the increase in A<sub>610nm</sub> of 0.1 in 1 hour and relative to a blanco sample.
ACTIVITY ASSAY GLYCOCEN BRANCHING ENZYME

The total activity of the enzyme was measured using the iodine assay\textsuperscript{21}, which is based on the formation of a blue complex between iodine-iodide and a linear $\alpha(1\rightarrow4)$ linked glucan of a certain length. The sum of the transglycosylation and the hydrolytic activity of the enzyme can be measured by monitoring the decrease in absorbance. The reaction mixtures contained 150 $\mu$L of 0.125% (W/V) amylose V (Avebe, Foxhol, The Netherlands) and 50 $\mu$L enzyme at the appropriate concentration (15 to 25 mg·mL$^{-1}$). At different time intervals, aliquots of 15 mL were taken and the reaction was terminated by addition of 1.5 $\mu$L of 1 M NaOH and 150 $\mu$L of iodine reagent (0.01% [W/V] I$_2$, 0.1% [W/V] KI). One unit of enzyme activity is defined as the decrease in absorbance of 1.0 per min at 660 nm for amylose.

SYNTHESIS OF MALTOHEPTAOSE

500 g of $\beta$-cyclohexextrin was dissolved in 2 L 0.01 M HCL and was refluxed for 2 hours. The mixture was neutralized with 1 M NaOH, slowly cooled to room temperature, and stored overnight at 4 °C. The precipitated $\beta$-cyclohexextrin was removed by means of filtration. 20 mL of $p$-xylene was added to the filtrate and heated to 60 °C for 1 hour. The filtrate was then slowly cooled to room temperature and stored overnight at 4 °C. The resulting $p$-xylene/$\beta$-cyclohexextrin complex was removed via filtration and the filtrate was concentrated to 200 mL via rotary evaporation. Again 20 mL of $p$-xylene was added to the filtrate and heated to 60 °C for 1 hour. The filtrate was cooled to room temperature and stored at 4 °C. The remaining $\beta$-cyclohexextrin was removed as $p$-xylene/$\beta$-cyclohexextrin complex via filtration. The filtrate was precipitated in 2 L cold ethanol and dried in vacuo. The resulting maltoheptaose appeared as an off-white powder in a yield of 10%.
SPECTROSCOPIC PHOSPHATE DETERMINATION

Preparation of the solutions:

1. (Reducing agent). 25 g Pyrosulfite and 1 g sodium sulfite were dissolved in 60 mL water R.O. 200 mg Metol was dissolved in 1 mL water R.O. The metal solution was added to the other solution and filled to 100 mL. This solution was stored in the dark.

2. (Buffer). 100 g Sodium acetate was dissolved in 250 mL water R.O.

3. (Complexing agent). 12.5 g Ammonium molybdate was dissolved in 100 mL water R.O. 125 mL 5 N sulphuric acid was added while stirring and filled with water R.O. to 250 mL.

Assay. A 10-100 µL aliquot was added to a 10 mL vial. 500 µL of solution 1. and 1000 µL of solution 2. were added and diluted with water R.O. After 10 min, 2 mL of solution 3. was added and the vial was filled to 10 mL. The absorption was measured after 30 min at a wavelength of 716 nm.

TYPICAL ENZYME CATALYZED TANDEM POLYMERIZATION

Maltoheptaose (0.5 mM), G-1-P (25-500 mM), phosphorylase (5 U.mL⁻¹) and GBE₆₆ (250 U.mL⁻¹) were mixed and filled to 5 mL with 3-(N-morpholino)propanesulfonic acid buffer (MOPS, pH 7.0, 50 mM). When only phosphorylase was utilized, a citrate buffer (pH 6.2, 50mM) was used. Different ratio’s G-1-P to primer were made by varying the G-1-P concentration. The solution depended on the enzymes used, was incubated at 37 °C (tandem polymerization) or 38°C (phosphorylase) and shaken. The released amount of phosphate was measured with a modified¹⁴ method of Fiske and
Subbarow\textsuperscript{13}. Upon reaching equilibrium conditions, the reaction was terminated by a heat treatment (5 min in boiling water). Denatured enzymes were removed by means of centrifugation. Dialysis and lyophilization of the remaining solution yields the (hyperbranched) polysaccharides.

**METHYLATION ANALYSIS**

*Preparation of 4.2 M sodium dimysyl.* 0.5 g sodium hydride was dissolved in 5 mL dry DMSO at 50 °C in an ultrasonic bath.

*Sample preparation.* 2 mg dried sample was dissolved in 0.5 mL dry DMSO at 70 °C.

*Methylation with CH$_3$I.* 250 $\mu$L sodium dimysyl was added to the sample and mixed overnight at room temperature. While cooling on ice, 250 $\mu$L CH$_3$I was added and mixed for 1 hour. The methyl iodide was removed with a stream of nitrogen at 40 °C and 1.5 mL dichloro methane was added to the methylated samples. The solution was extracted by adding 1.5 mL water R.O. whereby the water layer was discarded. This extracting procedure was repeated 5 times and at last the dichloro methane was removed with a stream of nitrogen.

*Hydrolysis with 2M TFA.* 200 $\mu$L TFA was added to the dry sample and boiled for 1 hour. TFA was removed at 60 °C with a stream of nitrogen.

*Reduction with NaBD$_4$.* 400 $\mu$L NaBD$_4$ was added to the sample. The mixture was carefully shaken for 3 hours and the reduced product was lyophilized.

*Acetylation.* 80 $\mu$L acetic acid, 1.2 mL acetic anhydride and 40 $\mu$L 70% perchloric acid was added to the sample. While cooling on ice 4 mL water R.O. and 80 $\mu$L 1-methyl imidazole was added. The solution was extracted with 1 mL dichloro methane. 10 $\mu$L of the PMAA solution was used for the GC-FID analysis.

**BCA ASSAY**

*Preparation.* Solution A. 13.575g Na$_2$CO$_3$, 6.05g NaHCO$_3$ and 0.475g Na$_2$BCA were dissolved in 250 mL water R.O. Solution B. 312.5 mg CuSO$_4$.5H$_2$O and 315 mg lysine were dissolved in 250 mL water R.O.

*Assay.* Solution A and B were freshly mixed before the assay. 100 $\mu$L sample was incubated with 100 $\mu$L of reagent for 60 min at 80 °C in an oven. The samples were cooled on ice and the absorbance was measured at 550 nm. A calibration curve was made of maltose in the range of 10 – 70 $\mu$mol.
ANTHRONE ASSAY

Preparation. 60 ml water R.O. and 15 ml ethanol was mixed and cooled on ice. 400 mg of anthrone was dissolved in 200 ml of pure H₂SO₄. This anthrone solution was carefully added to the water/ethanol mixture. The anthrone solution was stored at 4 °C.

Assay. 1 ml sample and 10 ml anthrone reagents were mixed with a vortex stirrer and incubated for 10 min at 100 °C. The samples were allowed to cool down to room temperature before the absorbance was measured at a wavelength of 620 nm. A calibration curve was made of maltose in the range of 100 – 1000 μM.

2.3 RESULTS AND DISCUSSION

2.3.1 Optimum conditions for combined biocatalysis

Enzymes are usually only active within a narrow pH range. The pH influences the ionization states of the amino acid residues in the active site and the ionization of the substrate, which can result in a reduced catalytic activity. When two enzymes are used in a 1-pot synthesis, a compromise has to be found in reaction circumstances in a way that both enzymes perform the best.

The activity of potato phosphorylase as well as the activity of GBE₁₂₀ are measured with the corresponding activity assays, as described in the EXPERIMENTAL section, in different buffer systems with various pH values. The optimum activity of potato phosphorylase was found at a pH of 6.2 and the optimum activity of GBE₁₂₀ was found at a pH of 8.0 (see FIGURE 2.5). Therefore, the 1-pot systems, in which both enzymes are employed, are performed in a MOPS buffer at a pH of 7.0. Both enzymes were in this environment still active enough to catalyze the specific reactions.
FIGURE 2.5: (○) Activity profile of potato phosphorylase and (△) GBE<sub>DG</sub> at different pH values.

The temperature range in which both enzymes are active is, like the pH, restricted to a small bandwidth. The optimum temperature for GBE<sub>DG</sub> was found to be 32 °C while the optimum temperature for the potato phosphorylase was 38 °C (see FIGURE 2.6).

The combined biocatalysis is performed with good results at a temperature of 37 °C.

FIGURE 2.6: (○) Activity profile of potato phosphorylase and (△) GBE<sub>DG</sub> between 30 and 55 °C.
2.3.2 Visual appearance of the reaction products

**FIGURE 2.7** shows the difference between a polymerization performed in the presence of phosphorylase and GBE_{DG} (LEFT) and a polymerization performed in the presence of phosphorylase alone (RIGHT).

![Figure 2.7: Reaction vials after polymerization.](image)

The vial on the LEFT is the result of the combined catalyzed polymerization in which phosphorylase and GBE_{DG} are employed. The resulting hyperbranched polysaccharide (DP \(~\sim\)500) forms a stable opaque solution. The vial on the RIGHT is the result after incubation with phosphorylase alone. The synthetic, linear, amylose (DP \(~\sim\)500) precipitates during polymerization out of solution. This experiment gives us a visual proof that the combined action of the enzymes indeed produces a branched polyglucan, as only the branched structure is soluble.

In the following section a more detailed characterization of the obtained structures is described.

2.3.3 Kinetics of the phosphorylase catalyzed reaction

The phosphorylase catalyzed reaction behaves like a living polymerization including all following characteristics:\(^8\):

- The polymer chain grows linear with time.
- Addition of G-1-P results in re-growth of the polymer chain.
- The termination step is absent.
- Polymers with a low polydispersity are obtained\(^22\).
Since the phosphorylase driven reaction is an equilibrium reaction the linearity of monomer consumption diminishes when the concentration of G-1-P decreases (see Figure 2.8). In equilibrium about 70% of the G-1-P is consumed, corresponding to an equilibrium constant (K) of 2.3.

![Graph showing the reaction course of a phosphorylase driven polymerization.](image)

**Figure 2.8:** Typical reaction course of a phosphorylase driven polymerization. Reaction performed in 10 mL citrate buffer (pH 6.2, 50 mM) at 30 °C with a primer concentration of 2 mM, 360 mM G-1-P and 0.1 U.mL⁻¹ phosphorylase.

The molecular weight of the synthetic amylose is solely dependent on the feed ratio G-1-P, analogue to a conventional anionic polymerization where the ratio of monomer over initiator determines the molecular weight. The degree of polymerization can be determined following Equation 2.4.

\[
\text{Degree of polymerization} = \frac{[G-1-P]}{[\text{maltoheptaose}]} + 7
\]

where 7 represents the amount of glucose residues from the primer.

The initial reaction speed depends on the amount of potato phosphorylase and the concentration of primer molecules. In a later stage of the reaction, the decreasing concentration of monomer will become rate limiting.
When, next to the potato phosphorylase, \( \text{GBE}_{\text{DG}} \) is employed the reaction speed is no longer only dependent on these two parameters as \( \text{GBE}_{\text{DG}} \) introduces new branches in situ. Each newly introduced branch acts as an intramolecular primer site and increases the available points for polymer growth.

**FIGURE 2.9** shows a phosphorylase driven reaction and is compared with a reaction in the presence of \( \text{GBE}_{\text{DG}} \). In this particular case no difference in reaction speed is seen as phosphorylase is present in a rate limiting concentration. The increase in primer sites (as catalyzed by \( \text{GBE}_{\text{DG}} \)) can not be utilized completely by phosphorylase due to the rate limiting concentration of the enzyme. The advantage of this strategy is that the reaction products of the phosphorylase driven reaction and the combined biocatalysis can be compared at any time during polymerization. In terms of chemical structure and molecular architecture, the only difference is the branched character.

![Figure 2.9: The reaction course of a phosphorylase driven polymerization with (○) and without \( \text{GBE}_{\text{DG}} \) (□).](image)

**FIGURE 2.10** shows the result of the obtained degree of polymerization in equilibrium conditions of reactions incubated with a fixed primer concentration and different feed ratio’s of G-1-P. As shown in **EQUATION 2.4**, the degree of polymerization correlates linearly with the feed ratio G-1-P. This is experimentally validated by the results as depicted in **FIGURE 2.10**. When \( \text{GBE}_{\text{DG}} \) is utilized, the same
correlation between the degree of polymerization and the feed ratio G-1-P is observed.

![Graph showing degree of polymerization vs. feed ratio G-1-P]

**FIGURE 2.10:** Degree of polymerization for different phosphorylase driven polymerizations (□) without GBE\(_{DG}\) and (○) with GBE\(_{DG}\) versus varying G-1-P feed ratios. The slope of the linear trend line represents the conversion of G-1-P and corresponds to 70%. Reactions performed in 0.5 mL MOPS buffer (pH 7.0, 50 mM) at 37 °C with a primer concentration of 0.5 mM, 25-250 mM G-1-P, and 5 U.mL\(^{-1}\) phosphorylase.

In conclusion, when the right reaction conditions are chosen no difference is seen in the reaction course. Both the phosphorylase driven reaction and the combined catalyzed reaction show the same kinetics. However, there is a difference with respect to the molecular architecture as the combined biocatalysis yields water soluble branched polysaccharides. By controlling the feed ratio of G-1-P, predetermined sizes of linear and branched structures are obtained depending on the enzymes used.

### 2.3.4 Degree of branching

During the enzyme catalyzed branching the primer molecule grows due to the action of phosphorylase. *In situ* branch formation is realized by the utilization of GBE\(_{DG}\). The minimum substrate length for GBE\(_{DG}\) is expected to be around 20 α(1→4) linked glucose residues. This means that phosphorylase is in the initial stage of the reaction
the only active enzyme. When the growing primer exceeds the length of 20 glucose residues, GBE\textsubscript{DG} is able to catalyze branch formation.

**THEORETICAL DEGREE OF BRANCHING**

The maximum degree of branching (DB) of polysaccharides as obtained via the combined biocatalysis correlates with the preferred glucan chain length (c.l.) that is transferred by GBE\textsubscript{DG} (see Equation 2.5).

\[
\lim_{\text{DP} \rightarrow \text{c.l.}} \text{DB}(\%) = \left( \frac{1}{\text{chain length}} - \frac{1}{\text{degree of pol.}} \right) \times 100\%
\]

2.5

**FIGURE 2.11** depicts the theoretical evolution of the average degree of branching with growing degree of polymerization as calculated by Equation 2.5. Three different average side chain lengths (DP 8, 9 and 10) are shown together with the corresponding maximum attainable degree of branching (dashed line in Figure 2.11).

**FIGURE 2.11**: (○) theoretical average side chain length of 8 glucose residues; (□) theoretical average side chain length of 9 glucose residues; (△) theoretical average side chain length of 10 glucose residues.

Branching enzymes transfer a range of glucans with different chain lengths rather than one specific chain length. However, the proposed evolution of the degree of
branching should apply to the combined biocatalysis if the branching enzyme affinity to the donor substrate stays constant during the course of the reaction.

**EXPERIMENTAL DETERMINATION OF THE DEGREE OF BRANCHING**

To obtain the course of branching as a function of time of the combined biocatalysis, the reaction was followed by methylation analysis and the combined BCA/anthron assay. The RSD assay results are considered as not accurate and are discarded since the samples were not G-1-P free. These results together with the evolution of the degree of polymerization in time is plotted in **Figure 2.12**.

![Figure 2.12](image)

**Figure 2.12**: (○) Evolution of the degree of polymerization in time. Evolution of the degree of branching in time measured via (○) BCA/anthrone assay and (△) methylation procedure. All dashed lines are guide lines to the eye.

The degree of branching levels of to a value of 15.7 % and 6.7 % for, respectively, the BCA/anthron assay and the methylation analysis. The degree of branching does reach the expected plateau (as shown in **Figure 2.11**) but the difference between the two methods is rather substantial. Both the methylation analysis and the combined BCA/anthrone assay consists of a complex and laborious multi-step procedure which makes this method susceptible to inaccurate values due to accumulated experimental errors.
An alternative method is found in $^1$H-NMR spectroscopy. By taking the ratio of the $\alpha(1\rightarrow6)$ and $\alpha(1\rightarrow4)$ signal, the degree of branching can be calculated in a fast and accurate manner.

DETERMINATION OF THE DEGREE OF BRANCHING BY $^1$H-NMR

In the next section, the degree of branching is determined with $^1$H-NMR spectroscopy. Again, the degree of branching and the degree of polymerization were followed during the course of a tandem polymerization. FIGURE 2.13 shows the combined results.

FIGURE 2.13: (□) Evolution of the degree of polymerization in time. (○) Evolution of the degree of branching in time measured via $^1$H-NMR. Dashed lines are guide lines to the eye.

FIGURE 2.13 nicely illustrates that the degree of branching is independent of the degree of polymerization at higher molecular weights. This is in accordance with the theoretical calculated asymptote as shown in FIGURE 2.11.

The initial part of the reaction course does not show the expected increase in the degree of branching, instead values of around 11% are measured during the complete course of the reaction. This is caused by the fact that measurements of the
degree of branching of samples with a DP<50 is difficult due to the necessary dialysis step in order to purify the sample.

The experimentally obtained data is fitted with the proposed theoretical evolution of the degree of branching in the case of an average side chain length of 9 glucose residues (see **FIGURE 2.14**).

![Graph](image)

**FIGURE 2.14:** (□) Experimental data as determined with $^1$H-NMR. (dashed line) Theoretical average degree of branching on the basis of a side chain length of 9 glucose residues.

On the basis of these results it is concluded that the hyperbranched products have on average side chain lengths of 9 glucose residues. However, branching enzymes transfer a range of side chains to the $\alpha(1 \rightarrow 6)$ positions rather than one specific length. Therefore the side chain length pattern is further elucidated with MALDI-ToF experiments.

**DETERMINATION OF THE SIDE CHAIN LENGTH DISTRIBUTION BY MALDI-TOF**

Matrix assisted laser desorption/ionization – time of flight (MALDI-ToF) mass spectrometry allows the mass analysis of biopolymers via a soft ionization technique. The technique requires a suitable matrix in which the analyte is mixed. Upon irradiation the matrix/analyte mixture with short laser pulses, ionized analyte molecules are co-desorbed with the matrix and are accelerated and analyzed by a ToF-MS detector.
It is rather difficult to ionize large neutral polysaccharides such as amylose and the branched structures as synthesized in this chapter. Therefore it is not possible to directly determine the mass with MALDI-ToF. However, linear α-glucans with a DP below 30 can be detected.

Therefore synthesized hyperbranched polysaccharides are debranched with the enzyme isoamylase. With this approach it becomes possible to determine the side chain length distribution with MALDI-ToF as isoamylase selectively catalyses the hydrolysis of α(1→6) linkages. The resulting mixture of linear oligosaccharides is measured with MALDI-ToF spectroscopy (see FIGURE 2.15).

![Figure 2.15](image1.png)

**FIGURE 2.15:** The followed procedure in order to obtain the side chain distribution with MALDI-ToF spectrometry.

$^1$H-NMR spectroscopy is used to ensure complete hydrolysis of all α(1→6) linkages. As can be seen in FIGURE 2.16, the α(1→6) signal at 5.0 ppm is indistinguishable from the debranched product, proving that the selective hydrolysis of the α(1→6) linkages has gone to completeness.

![Figure 2.16](image2.png)

**FIGURE 2.16:** A) α-glucan after debranching with isoamylase, B) branched α-glucan.
**FIGURE 2.17** shows the MALDI-ToF spectrum of a debranched polyaccharide. The mixture of resulting oligosaccharides after debranching gives a rather narrow distribution of side chains with a DP between 4 and 15 glucose residues, with a preference for oligosaccharides with a length of 6-8 glucose residues.

**FIGURE 2.17:** Chain length distribution profile of a debranched polysaccharide with an original DP of 600. The relative difference between signals is 162 m/z and corresponds to the mass of 1 glucose residue.

Palomo et al.\textsuperscript{23} incubated amylose V with GBE\textsubscript{DG} and determined the side chain pattern with high performance anion exchange chromatography with a pulsed amperometric detector (HPAEC-PAD). The resulting distribution consisted of side chains with a DP between 4 and 17 glucose residues, with a preference for oligosaccharides with a length of 6-7 glucose residues.

The results as obtained here by MALDI-ToF fit well with the average side chain length as obtained by \textsuperscript{1}H-NMR and with the results published by Palomo et al.
2.4 CONCLUSION

The one pot synthesis of a hyperbranched \( \alpha \)-glucan via an enzyme catalyzed tandem polymerization is demonstrated. The production of hyperbranched structures includes glucose-1-phosphate (G-1-P) as donor substrate, maltoheptaose as primer and the enzymes phosphorylase and the branching enzyme of *Deinococcus geothermalis* (GBEDG). Potato phosphorylase is able to catalyze the linear \( \alpha(1\rightarrow4) \) chain growth of maltoheptaose at the non-reducing site using glucose-1-phosphate as donor substrate while GBEDG catalyses the in situ branch formation by a transglycosylation reaction in which an \( \alpha(1\rightarrow4) \) linkage of the donor substrate is cleaved, followed by an attachment of the released oligosaccharide to an acceptor substrate via an \( \alpha(1\rightarrow6) \) linkage.

The course of the reaction can be followed by UV spectroscopic measurement of the released organic phosphate by complexing it with molybdate. Via this technique, the degree of polymerization and conversion of the reaction can be determined during the course of reaction. In general 70 % of the G-1-P is converted. The size of the hyperbranched polysaccharide can be predetermined by controlling the feed ratio of G-1-P which acts as the donor substrate.

A fast and accurate determination of the degree of branching as well as the determination of the average side chain length is achieved with \(^{1}H\)-NMR spectroscopy. The maximum attainable degree of branching is independent of the degree of polymerization and is 11 %.

The side chain distribution is determined with MALDI-ToF. The side chain distribution consists of rather short chains with a narrow side chain distribution. The distribution proves that GBEDG preferentially transfers oligosaccharides with a length of 6 to 9 glucose residues. This length is also optimal for the phosphorylase catalyzed chain growth. Moreover, it is known that short side chains retard the retro-degradation process and enhance the water solubility\(^{24}\). These are important features for the use of hyperbranched polysaccharides in the biomedical field and the food industry.

The hyperbranched polysaccharides have a degree of branching and side chain distribution comparable to amylose V samples that are incubated with GBEDG. However, the procedure described in this chapter allows complete control over the degree of polymerization.
2.5 REFERENCES
