Master Thesis

Oligosaccharide Analysis

Assessment of the DNS Method and Specification of Glucans Processed with CGTase

By

Matilda Johansson

Department of Biotechnology
Lund university
Sweden
March 10th, 2020

LUND UNIVERSITY

Supervisors: Carl Grey and Stefan Ulvenlund

Examiner: Patrick Adlercreutz

Preface

The project was executed between August 26th 2019 and March 30th 2020 in Enza Biotech's facilities. Thanks to a special appearance of SARS-CoV-2, the last alterations of the report were, however, made at the home office.

The project was initiated based on a need to gain a deeper understanding of the activity of cyclodextrin glycosyltransferase (CGTase), and specifically how the product profile is affected by the type of glucan substrate used and by the presence of a surfactant.

I want to thank Stefan Ulvenlund, Johan Sander, Maru Viloria, Magdalena Axenstrand, Marie Wahlgren, Mia Andersson, Carl Grey and Patrick Adlercreutz for the support and dedication that went into helping me finish this project. It was a pleasure working with you.

Abstract

In this study, the reagent 3,5-dinitrosalicylic acid (DNS) and high-performance anion exchange chromatography (HPAEC) were used for carbohydrate characterisation. It was revealed that the DNS reaction is not exclusive to reducing ends as commonly thought, but rather that the reagent reacts with other parts of the carbohydrate molecules as well. HPAEC analysis was used to find how the product distribution is affected when cyclodextrin glycosyltransferase (CGTase) utilizes different substrates, and what effect an inert surfactant has on the outcome. The studies were performed in cooperation with Enza Biotech.

The DNS method has been used for over a century to detect and quantify reducing ends of carbohydrates, as the reaction between a sugar and the DNS molecule is commonly thought to be exclusive to the reducing end aldehyde group. However, through analysing standard curves of maltooligosaccharides of different degrees of polymerization (DP), it was found that DNS molecules were reduced through reactions with other hydroxyl groups as well. Further studies are needed to find how specific this result is to the protocol used in this study.

CGTase is a multifunctional enzyme with the unique ability to cyclize oligoglucans into cyclodextrins (CDs). In addition, it catalyses hydrolysis and two more transglycosylation reactions, all performed on α-1,4-glycosidic bonds. CGTase was used with four different substrate setups, namely α-CD (DP 6), maltodextrins of DEs (dextrose equivalents) 1 and 17, and DE 1 maltodextrin in combination with an inert surfactant. The reactions were run for 48 hours and speciation was performed by HPAEC-PAD analysis.

The product profiles shared similar traits but differed in a few key areas. A higher average DP was observed within the linear fraction of the reaction where α-CD was used as raw material. The reaction with the shorter maltodextrin stabilized fast and showed close to no net change, while the reaction with the longer maltodextrin showed changes in the product profile for the duration of the experiment and reached a lower DP. There was a strong indication that the presence of the inert surfactant led to the formation of inclusion complexes between the surfactant and the CDs. In general, all reactions finished with a major linear and a minor cyclic glycan fraction. The distribution of glucan species within the linear fractions of all reactions were distributed according to what resembled Flory statistics, a molecular weight distribution often observed in polymerization processes.

Table of Content

Nomenclature List

Abbreviation	Meaning
ACN	Acetonitrile
ANS	3-amino-5-nitrosalisylic acid
APG	Alkyl polyglucoside
CAD	Charged aerosol detector
CD	Cyclodextrin
CGTase	Cyclodextrin glycosyltransferase
DE	Dextrose equivalent
DNS	3,5-dinitrosalisylic acid
DP	Degree of polymerization
EO	Polyoxyethylene
HPAEC	High-performance anion-exchange chromatography
HPLC	High-performance liquid chromatography
MQ	Milli-Q water
MS	Mass spectrometry
NaAc	Sodium acetate
NaOH	Sodium hydroxide
PAD	Pulsed amperometric detection

1. Introduction

Cyclodextrin glucosyltransferase (CGTase; EC 2.4.1.19) is an enzyme that acts exclusively on the α-1,4-glycosidic bonds of glucans. It catalyses four different reactions (Enzyme.expasy.org, 2020). In the unique cyclization reaction, a maltooligosaccharide is cleaved and then cyclized to form a cyclodextrin (CD) molecule consisting of six or more glucose repeating units. The coupling reaction is the opposite reaction, where a CD is cleaved and the resulting linear fragment attached to an acceptor molecule. In the disproportionation reaction, a part of a maltooligosaccharide is transferred onto an acceptor molecule, and in the hydrolysis reaction, a glycoside bond is cleaved with the addition of a water molecule.

Enza Biotech use CGTase to extend the hydrophilic head groups of alkyl glucosides to create alkyl polyglucosides (APGs), with maltodextrin as glycosyl substrate. However, as the enzyme is multifunctional, the reaction results in an array of oligosaccharide species. Analysis of the oligosaccharide profile is key in understanding how different parameters affect the resulting product distribution, which is important for understanding how to improve yield and cost effectiveness of the process.

High-performance anion-exchange chromatography (HPAEC) is a well-established method for quantitative carbohydrate analysis. The method enables detection of low concentrations, identification of carbohydrate species and allows for the addition of a mass spectrometer (MS) (Rohrer, 2013). However, the method is time consuming and the equipment is advanced and expensive. Another common method for analysis of carbohydrates is the DNS method. It is less sensitive but requires a simpler instrument. It is a colorimetric method that has been used for over a century to detect reducing sugars (Sumner and Graham, 1921). 3,5-dinitrosalisylic acid (DNS) is reduced to 3-amino-5-nitrosalisylic acid (ANS) through a redox reaction with the reducing sugar, causing a colour shift from yellow to red-brown (Miller, 1959). The change in absorbance is measured and used to quantify the amount of reducing sugars.

The aim of the present project was to elucidate the product profile after enzymatic digestion of different glucans by CGTase. The effect of an inert surfactant was also tested. Knowledge of the product distribution is important when designing different elements of a process, such as the choice of raw materials and downstream processing. To do this, instrument and process methods for the HPAEC were developed and optimized with particular emphasis on resolution and detection of longer oligosaccharides and of CDs. In addition, the DNS method was evaluated to learn if it was suitable as a validating method to be used in parallel to the HPAEC.

2. Literature Study

2.1. CGTase

The four CGTase reactions (Figure 1) are catalysed through the same double displacement mechanism, going through a covalently bound enzyme-substrate intermediate (McCarter and Withers, 1994). The different types of reactions are defined by what kind of substrates are used, and the products that are formed. The three transglycosylation reactions are α-retaining, resulting in a preserved stereochemistry in the products.

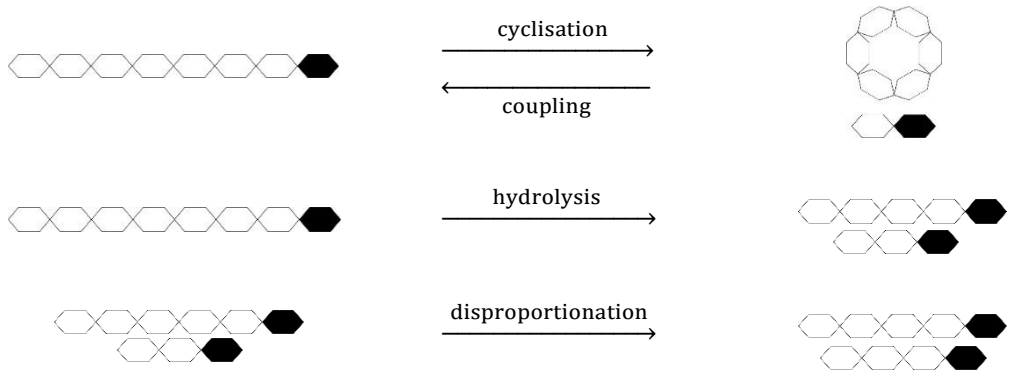


Figure 1. The four reactions catalysed by CGTase. The donor molecule must have a DP of at least five, and the acceptor must have an available hydroxyl group. Image created in Photoshop CS5.

All four reactions require a glucosyl donor with a degree of polymerization (DP) of at least five (Penninga et al., 1995). The active site has a cleft topology and is divided into nine subsites, each with the ability to bind one glucose repeating unit (Bender, 1990). The subsites are labelled -7 to +2 as suggested by Davies et al. (1997), see Figure 2. The cleaved bond (scissile bond) is located between subsites -1 and +1 with negative values toward the non-reducing end of the substrate.

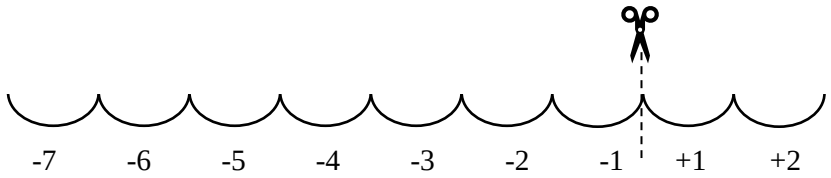


Figure 2. CGTase active site layout. Each subsite can bind one glucose repeating unit. Image created with Word 365.

While several amino acids are engaged in the reaction through hydrogen bonds, (Strokopytov et al., 1995), the actual reaction is facilitated by a catalytic trio. Asp^{229} (numbering refers to a CGTase from *Bacillus circulans*) acts as a nucleophile, Glu^{257} as both acid and base, and Asp^{328} stabilizes the transition state (Klein et al., 1992, Mosi et al., 1997, Strokopytov et al., 1995).

The first part of the reaction, the cleaving of the scissile bond, is identical for all four reactions. Asp^{229} makes a nucleophilic attack on the anomeric carbon (C1) of the donor substrate while Glu^{257} acts as an acid and donates a hydrogen. As the scissile bond is broken and the leaving group is gone, subsites +1 and +2 are left empty and the acceptor molecule binds. This time, Glu^{257} acts as a base and attracts the hydrogen of the acceptor. The acceptor then attacks the covalent intermediate and is linked to the donor residue, thus restoring the enzyme to its initial state (Uitdehaag et al., 2002).

The main CGTase CD products consist of six, seven and eight glucose repeating units and are referred to as α-, β- and γ-CD respectively. No smaller CDs can be formed, but larger CDs have been identified

in small amounts. Terada et al. (1997) found that CGTase could create CDs of DP of above 60, but that these large CDs were digested over time in favour for the smaller CDs previously mentioned.

The CGTase used in this study was Toruzyme® 3.0L, obtained from Novozymes A/S. It is a thermostable CGTase originally sourced from a *Thermoanaerobacter* sp., subsequently modified by the supplier and expressed in *Bacillus licheniformis*.

2.2. Enza Biotech

Enza Biotech are developing a range of sustainable non-ionic surfactants to be used in the health and personal care sectors. Surfactants are amphipathic compounds that lower the surface tension between two phases (Rosen and Kunjappu, 2012) and have a wide variety of applications. The Enza surfactants are produced through an enzymatic reaction with CGTase, using maltodextrin and alkyl glucosides as substrates. Maltodextrin is degraded starch and thus contains both α-1,4- and α-1,6-glycosidic bonds, as highlighted in Figure 3. The presence of α-1,6-bonds enables solubilisation of the substrate, but as CGTase cannot act on α-1,6-glycosidic bonds, this may limit the yield.

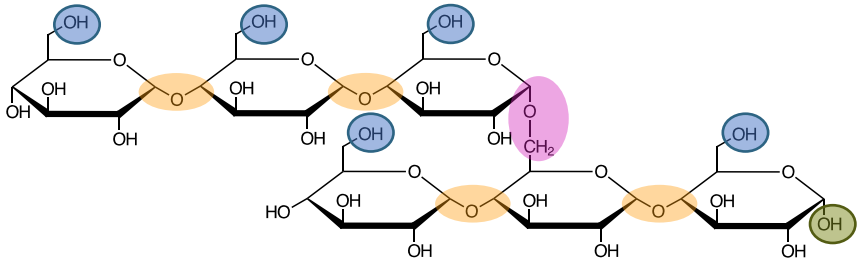


Figure 3. Maltodextrin molecule with the reducing end marked in green and the 6C in blue. The molecule is linked through four α-1,4-glycosidic bonds (orange) and one α-1, 6-glycosidic bond (purple).

The long hydrophilic head group, see Figure 4, makes the surfactant well suited for use in personal care and health care sectors. As the enzyme is used for elongation, the main interests of the company are the coupling and disproportionation reactions.

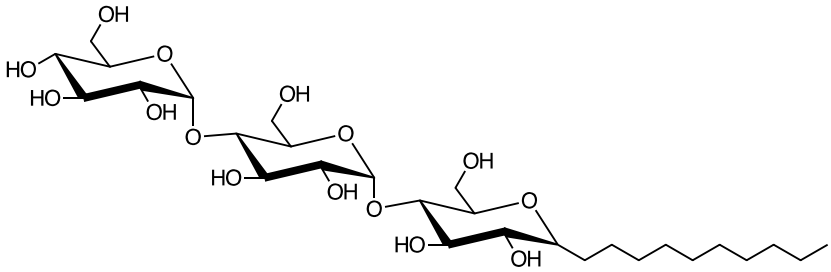


Figure 4. Example of an APG molecule. The number of glycosyl groups in the hydrophilic head group and the length of the hydrophobic chain can vary.

Apart from the desired surfactant product, the enzymatic processing also results in an array of by-products consisting of oligomeric "free" sugars, together with α- and β-CDs. However, the distribution and prevalence of these species in the reaction mixture are largely unknown. Analysis of products is made more difficult by complex formation. CDs and surfactants can form inclusion complexes through interactions between the CD and the hydrophobic part of the surfactant (Lin, 2001). The surfactant can also form inclusion complexes though interactions between the fatty chain and longer glucans.

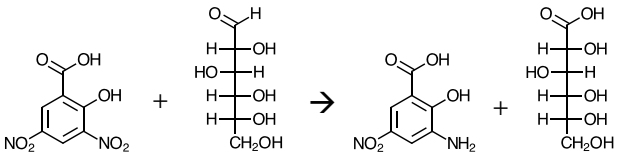
2.3. Carbohydrate Analysis

2.3.1. DNS Method/Assay

The DNS method has been used for detection of reducing sugars for close to a century (Sumner and Graham, 1921, Hübner, 1879). Reducing sugars contain either an aldehyde group (aldoses) or ketone group (ketoses). In the case of ketoses, a high pH opens the ring and creates an aldehyde group that provides a reducing end. Reducing sugars may be characterized in terms of dextrose equivalents (DE), which states the amount of reducing sugar as a percentage on a dry basis relative to glucose. This value can in turn be used to estimate the degree of polymerisation (DP), using the rule of thumb presented in Equation 1.

$$\mathrm{DP} = \frac{120}{\mathrm{DE}} \tag{1}$$

In 1921, Sumner and Graham published an article where they deemed the DNS method the best for detecting reducing sugar in urine samples. The method was further optimized by Miller in 1959. Since then, the method has been adapted to fit various applications. In the chemical reaction, 3,5-dinitrosalisylic acid (yellow) is reduced to 3-amino-5-nitrosalicylic acid (red-brown). The reaction is shown in Figure 5a and b, and the result is usually measured spectrophotometrically as the absorption at λ = 540 nm.

a) 

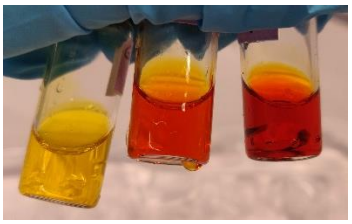
b) 

Figure 5. In the DNS reaction, DNS (pale yellow) is reduced to ANS (red-brown). The reaction on a a) molecular level and b) three samples with increasing concentrations of glucose from left to right.

Theoretically, two different reducing glucans in the same molar concentrations should generate the same response as the different molecules both carry one reducing end. However, it has previously been reported that different reducing sugars generate different responses (Saqib and Whitney, 2011).

2.3.2. High-Performance Anion-Exchange Chromatography

Detection and separation of carbohydrates through regular liquid chromatography is difficult as the molecules are polar and structurally similar. HPAEC was developed for carbohydrate analysis and is a method where the weakly acidic nature of carbohydrates is used for achieving separation of different species. Carbohydrates are partially ionized at a high pH, which causes them to interact with the stationary phase. As the ion strength of the eluent is increased, the carbohydrates are gradually eluted from the stationary matrix. The carbohydrates are detected through pulsed amperometric detection (PAD) where they are oxidized at the surface of a gold electrode. This generates a current that is measured to detect and quantify the carbohydrates, but it also adsorbs the analytes to the gold surface. To prepare the gold surface for the next measurement, the potential of the electrode is first increased to oxidize the entire surface, causing desorption of the analytes, and then lowered to reduce the surface to its original state (Rohrer, 2013).

2.3.3. Flory Distribution

The Flory-Schultz distribution is a statistical model often used to describe the distribution of linear polymers of different sizes in an ideal step-growth polymerization process (Flory, 1936). In a population adhering to Flory distribution, the logarithm of molar ratios, calculated as in Equation 2, shows a linear relationship through which the concentration of any species can be calculated if that of one is known.

$$\log(x_i) = \log\left(\frac{n_i}{n_{total}}\right) \tag{2}$$

Here, x_i is the molar fraction of carbohydrate molecules with DP i, n_i is the number of carbohydrate molecules with DP i and n_{total} is the total number of carbohydrates molecules.

3. Materials and Methods

3.1. DNS Assay

3.1.1. Materials

Chemicals used were DNS, NaOH, Rochelle salt (potassium sodium tartrate tetrahydrate), glucose, maltose, maltotriose, maltopentaose, maltooctaose and MilliQ-water.

A Hettich Benelux Multifunctional Thermo Shaker MHL 13 and a Shimadzu UV-1800 UV-VIS spectrophotometer were used for sample preparation and analysis.

3.1.2. Method

3.1.2.1. DNS Reagent Preparation

The DNS reagent was prepared according to a protocol provided by Sigma Aldrich, with minor modifications (Merck).

500 mL DNS reagent was prepared by dissolving 5.0 g of 3,5-dinitrosalisylic acid and 8.0 g NaOH in 150 mL of Milli-Q water at 60°C. 150 g Rochelle salt was added and some additional Milli-Q water to facilitate solubilisation, before topping up with Milli-Q water to reach a final volume of 500 mL. The reagent was stored in an amber glass bottle at room temperature.

3.1.2.2. DNS Sample Preparation

1 mL of sample was mixed with 0.5 mL of DNS reagent and boiled for 15 min at 100°C and 600 rpm in a Thermo Shaker. Samples were cooled down in an ice bath, then 300 µL of sample was diluted in 900 µL of Milli-Q water and absorbance was measured spectrophotometrically at 540 nm.

3.1.2.3. Standard Deviation

The standard deviation s of a data set was calculated according to Equation 3 as the square root of the variance.

$$s = \sqrt{\frac{\sum_{i=1}^{N}(x_i - \bar{x})^2}{N}} \tag{3}$$

x_i is the value of sample i, $\bar{x}$ is the average value of all samples and N is the number of samples.

3.2. Enzymatic Reaction

3.2.1. Materials

Chemicals used were Maltodextrin with DE 1 sourced from potato, maltodextrin with DE 17 sourced from maize, α-CD, Brij® L23 and CGTase (Toruzyme®, Novozymes). The enzyme was inactivated in a Hettich Benelux Multifunctional Thermo Shaker MHL 13 and the pH was monitored with VWR Dosatest pH test strips.

3.2.2. Method

The reactions were performed at 65°C and at 250 rpm, the starting materials used for the four reactions are presented in Table 1, the index expressing the glucan substrate and the presence or absence of the inert surfactant Brij® L23. Toruzyme® was used at 0.1 wt% in all four reactions. 500 µL samples were taken from each reactor according to Table 2.

Table 1. List of the four reactions, what substrate was used and if a surfactant was added. Toruzyme® was used at 0.1 wt% in all four reactions.

	$\mathbf{R_{CD-}}$	$\mathbf{R_{S-}}$	$\mathbf{R_{L-}}$	$\mathbf{R_{L+}}$
Substrate (5 wt%)	α-CD	Maltodextrin (DE 17)	Maltodextrin (DE 1)	Maltodextrin (DE 1)
Surfactant (1 wt%)	-	-	-	Brij® L23

Table 2. List of samples taken of the enzymatic reactions. The 0h samples were taken before the enzyme was added to the reactions, the remaining samples were taken after.

Sample	**Enzyme in sample**
0h	No enzyme
1min	Enzyme
15min	
30min	
45min	
1h	
2h	
9h	
24h	
48h	

The enzyme was inactivated by placing samples in the Thermo Shaker at 95°C at 600 rpm. In a first set of enzymatic reactions, the inactivation was carried on for 2.5 h. This proved to be too short, as repeated analysis of the same samples revealed a modified product profile with signs of residual enzymatic activity (see chromatogram in Appendix 7.1). This indicated that the thermostable enzyme was still active in the sample vials, digesting the material in the samples while they were queued for analysis. These results were therefore excluded from the report, with the exception of the surfactant analysis in section 4.2.1. For the reactions included in this report, the inactivation was carried out overnight (>16 h) and showed relatively stable product profiles (data not shown).

3.2.2.1. Molar Fractions

The molar fractions, x_i, used throughout the report were calculated as in Equation 4. Non-cyclic compounds were categorized according to their DP.

$$x_i = \frac{n_i}{n_{tot}} \tag{4}$$

Here, n_i is the amount of moles detected of the compound or group of compounds and n_{tot} was either the total amount of compounds found in the sample or the total amount of a group of compounds.

3.3. HPLC-MS

HPLC-MS was used for characterization of the surfactant and for surfactant analysis of R_{L+} samples.

3.3.1. Material

Chemicals used for HPLC were acetonitrile (ACN) and Milli-Q water. The details of the HPLC-MS (high performace liquid chromatography-mass spectrometry) system used for surfactant analysis are listed in Table 3.

Table 3. HPLC-MS instrument details.

HPLC System	
Analytical Column	Thermo Scientific Acclaim RSLC 120 C18 Length 150 mm Inner ⌀ 2.1 mm Particle size 2.2 µm Pore ⌀ 120 Å
HPLC System	Thermo Scientific™ UltiMate™ 3000 RS
Detector	Corona™ Veo™ Charged Aerosol Detector (CAD)
MS system	Thermo Scientific™ ISQ™ EC Single Quadrupole Mass Spectrometer
Software	Thermo Scientific™ Chromeleon™ 7.2.9 Chromatography Data System

3.3.2. Method

Surfactant analysis was performed at 0.400 mL/min and a column temperature of 40°C. The instrument method is detailed in Table 4. The inverse gradient was run with the opposite gradient; 90% - 70% acetonitrile with 0.350 min time delay. Samples were diluted 10x before HPLC analysis.

Table 4. Instrument method for surfactant analysis with HPLC-CAD.

Time (min)	Flow (mL/min)	Mobile Phases	
		ACN (%)	Milli-Q (%)
-3	0.400	70	30
0	Run		
0	0.400	70	30
15	0.400	80	20
30	0.400	90	10
31	0.400	70	30
32	Stop Run		

3.4. HPAEC

3.4.1. Material

Chemicals used were sodium acetate (NaAc), sodium hydroxide (NaOH), Milli-Q water, glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose, maltooctaose, α-CD, β-CD and γ-CD. Details of the HPAEC-PAD system used for carbohydrate analysis are listed in Table 5. The detector used a combination pH-Ag/AgCl reference electrode.

Table 5. HPAEC-PAD instrument details. The length, inner diameter and particle size of the columns are listed.

HPAEC System Details		Column Details		
		Length (mm)	Diameter (mm)	Particle Size (µm)
Guard Column	Thermo Scientific™ Dionex™ IonPac™ NS1	35	4	10
Pre Column	Thermo Scientific™ Dionex™ CarboPac™ PA200	50	3	5.5
Analytical Column	Thermo Scientific™ Dionex™ CarboPac™ PA200	250	3	5.5
HPAEC System	Thermo Fisher Scientific Dionex ICS-5000$^+$ DC			
Detector	Thermo Scientific™ Dionex™ ED40			
Software	Thermo Scientific™ Chromeleon™ 7.2.9 Chromatography Data System			

3.4.2. Method

Standard curves of sugars and CDs were created by first preparing stock solutions of 1 mg/mL of each compound and then using these to create a calibration solution with 5 µg/mL glucose, maltose and maltotriose, 10 µg/mL maltotetraose, maltopentaose and maltohexaose, 20 µg/mL maltoheptaose and maltooctaose, 50 µg/mL α-CD and 100 µg/mL β-CD. The concentrations were chosen to generate peaks of similar areas. The solution was diluted 2x, 5x, 10x, 20x and 50x to create a set of calibration standards of different concentrations for the standard curves. A flow of 0.500 mL/min was used and the column temperature was set to 33°C. R_{CD-} samples were diluted 1000x before HPAEC analysis, whereas R_{S-}, R_{L-} and R_{L+} samples were diluted 500x.

The instrument method used for analysis of samples from the enzymatic reactions is detailed in Table 6. The pulse sequence used was Carbo quad. Calibration standards were run with an instrument method identical to the initial 35 min of this sequence, after which it was interrupted as all compounds were eluted at this point. The instrument method was optimized so as to achieve narrow and well separated peaks, as well as with respect to sensitivity. It successfully separated peaks of α- and β-CD from any major maltodextrin peaks, with α-CD eluting between maltotriose and maltotetraose and β-CD eluting between maltooctaose and maltononaose (see chromatogram in Figure 13). γ-CD seemed to fully adsorb to the guard column and could not be successfully identified through the analysis methods applied in this project. The compound was assumed to exist in small enough levels not to affect the analysis results.

Table 6. Instrument method for detection and quantification of glucans and α- and β-CD after CGTase.

Time (min)	Flow (mL/min)	Mobile Phases	
		400 mM NaOH (%)	400 mM NaOH, 100 mM NaAc (%)
-15	0.500	100	0
0	Run		
0	0.500	100	0
1	0.500	100	0
25	0.500	76	24
29	0.500	76	24
85	0.500	20	80
85.5	0.500	0	100
90.5	0.500	0	100
91	0.500	100	0

The detection parameters that were chosen as default in all Chromeleon chromatograms are shown in Table 7. All chromatograms were inspected and, when necessary, manually altered to ensure consistent integrations.

Table 7. Detection parameters used in Chromeleon 7.2.9 for both standards and samples.

Retention Time	Parameter	Parameter value
<Initial>	Consider Void Peak	Off
<Initial>	Smoothing width	0.5
<Initial>	Baseline Noise Range	0.430…1.862
0	Snap Baseline On	
0	Inhibit Integration	On
2.009	Inhibit integration	Off
83.933	Inhibit Integration	On

3.5. Softwares

Microsoft Excel 2016 was used to review raw data and create graphs. Molecular structures were created in ChemDraw. Photoshop CS5 was used to create illustrations.

4. Results and Discussion

4.1. DNS

The DNS method was evaluated as a potential analysis method for parallel validation of the average DP of a carbohydrate sample found though the HPAEC results. It is a spectrophotometric assay that allows fast analysis of multiple samples. As the method is designed to specifically detect the reducing ends of reducing sugars, for instance maltodextrins, it was assumed to be an efficient way to detect the number of non-cyclic carbohydrate compounds in a sample. Coupled with a spectrophotometric cyclization assay and the known mass concentration, it is potentially an alternative method of calculating the average DP within the linear glucan fraction.

The suitability of the method was evaluated by examining the effect of a few parameters and on the findings after running different calibration standards within the homologous series of maltooligosaccharides. Based on the results from these runs, the DNS method was considered unfit for analysis of the enzymatic reactions, as will be elaborated on below.

4.1.1. Reagent Solutions

The DNS reagent was first prepared according to the Sigma Aldrich protocol. As there was little or no colour development when the reagent was used with glucose samples, the pH was increased to 14 by titration of NaOH. Although this reagent solution did generate a response, it was discarded due to precipitation in the storage flask. Notably, as the highest pH used for calibration of the pH electrode was 10, the final apparent pH may have been inaccurate. If the necessary amount of NaOH had instead been calculated, rather than measured, this reagent solution might have proved functional.

A new reagent solution was successfully made with a modified recipe that contained lower amounts of both DNS and NaOH, but with an altered acid-to-base ratio (see section 3.1.2 for details on composition). In relation to the amounts used to prepare the first reagent solution, the amount of DNS was decreased more than that of NaOH, which resulted in a higher pH than in the first solution despite the lower nominal concentration of NaOH. This could explain why there were no issues with the second reagent.

4.1.2. Heating Time

As a test of how critical the heating time during sample preparation was, a set of samples were heated for just one minute, in parallel to a set of samples heated for 15 minutes as dictated by the protocol. No spectrophotometric measurements were made as it was clear from visual comparison that the colour had not developed fully after the shorter reaction time. However, the next day, the colours of the samples had deepened to seemingly match those of the corresponding samples that had been heated for 15 minutes. This may be taken as an indication that the DNS reaction acts though a heat-activated cascade. More tests would be needed to confirm this theory, both to replicate the results and to identify any reaction intermediates.

4.1.3. Calibration Standards

4.1.3.1. Calibration Standards

Standard curves were made for glucose, maltose and maltotriose in triplicates, and for maltopentaose and maltooctaose in single measurements. The absorbance plotted against the molar concentration showed an increase in response for the longer sugars (Figure 6), seemingly indicating an increased reducing ability with increased DP. The increase in absorbance caused by increased sugar concentrations, i.e. the slope values from Figure 6, were plotted against the DP of each compound

(Figure 7). This revealed that the contribution from each glucose repeating unit followed a linear relationship.

This linearity indicates that each glucose repeating unit actually contributed with a fixed number of reducing groups, in addition to the reducing end group.

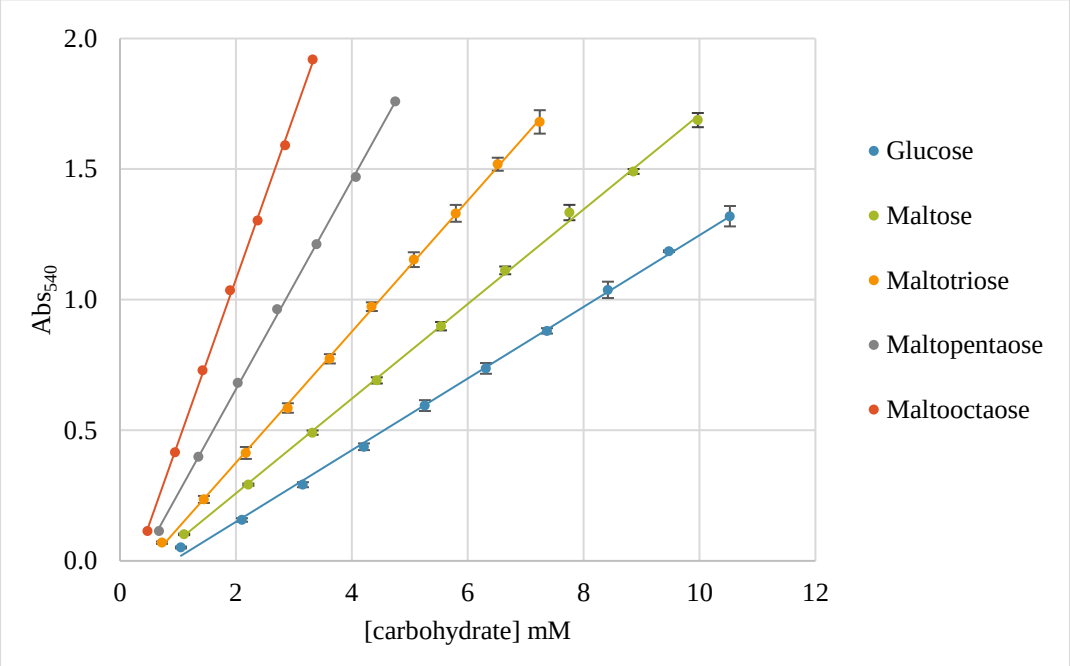


Figure 6. DNS results for glucose, maltose, maltotriose, maltopentaose and maltooctaose. The three shorter were run in triplicates. The response per mole increased with increased chain length.

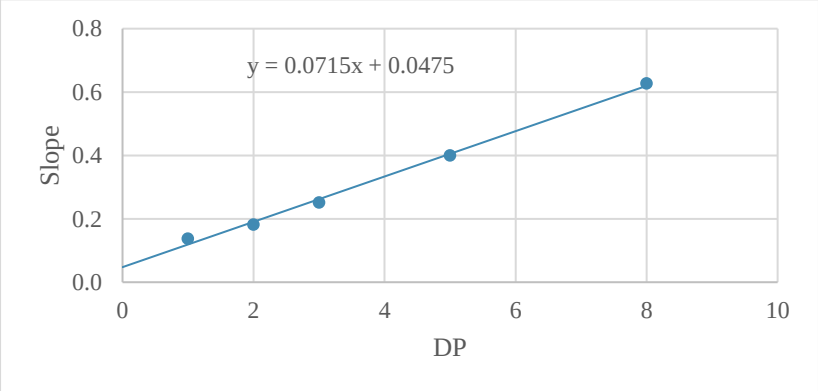


Figure 7. The slopes of each compound in Figure 6 plotted against their respective DP to visualize the effect each glucose repeat unit had on the reducing ability of the compound. The linear relationship suggests that a fixed number of reducing groups was contributed by each subunit.

4.1.3.2. Effects Caused by Individual Glucose Repeat Units

To investigate if the individual glucose repeating units within the different compounds contributed with the same amount of reducing ability, the absorbance of each carbohydrate reference standard was plotted against the concentration of glucose repeat units (Figure 8). This was calculated as in Equation 5.

$$[\text{glucose repeat units}] = [\text{carbohydrate}] * \text{DP}_{\text{carbohydrate}} \quad (5)$$

The trend of a decreasing slope with increased DP seen in Figure 8 showed that in longer maltooligosaccharides, each individual glucose repeat unit contributed less to the total level of

absorbance. As visualized in Figure 9, the slope values of longer maltooligosaccharides seemed to close in on an asymptote. These results led to the hypothesis that, in addition to the C1 reducing aldehyde group, at least one of the hydroxyl groups acted as reducing unite as well. As the C6 hydroxyl group is a primary alcohol and thus more susceptible to oxidation, it was assumed the most likely group to be involved in the reactions. If this was true, then the intercept in Figure 7 would represent the contribution of the reducing ends and the slope would represent the contribution from the C6 hydroxyls. The fact that these values were not the same indicated that there was a difference in reducing ability between the C1 aldehyde and C6 hydroxyl.

The results also demonstrated that the increased signal was not due to alkali degradation of the maltooligosaccharides. If alkaline hydrolysis of glycoside bonds had been the cause, the pattern would have been the reverse and closed in on glucose. In addition, it was deemed unlikely that random degradation would have caused results this consistent and reproducible.

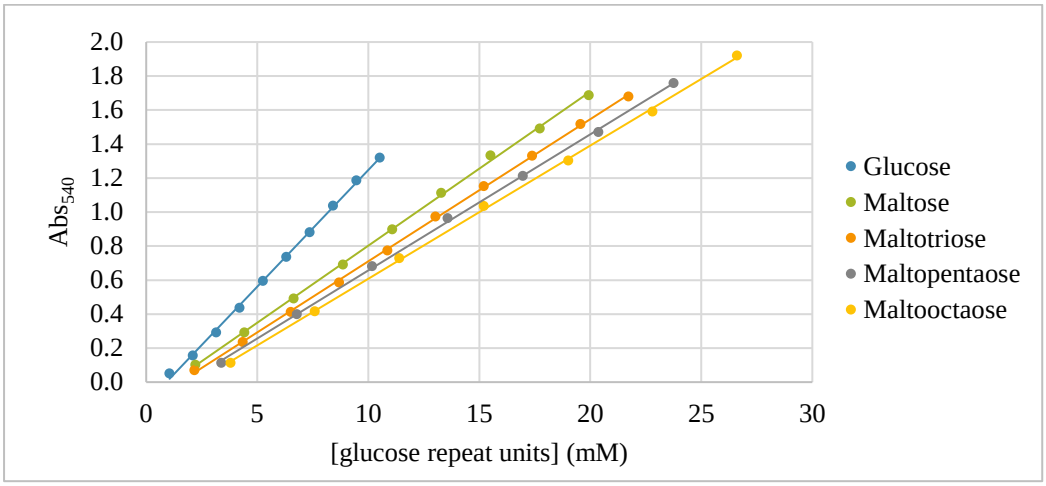


Figure 8. Absorbance plotted against the concentration of glucose repeat units.

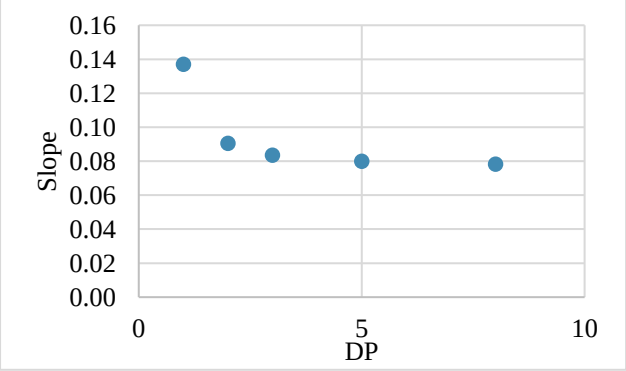


Figure 9. The slopes of the compounds in Figure 8 plotted against their respective DP to visualize the reducing contribution by each glucose repeat unit. The slopes of longer maltooligosaccharides seems to approach an asymptote.

4.1.3.3. Each Reducing Unit

If assumed that the C1 aldehyde and C6 hydroxyl did not contribute with the same amount of reducing ability as indicated in Figure 7, then the response factor, rf_{DNS}, of each carbohydrate would be calculated as in Equation 6 and the apparent concentration of redox reactions, $[redox]_{app}$, as in Equation 7.

$$rf_{DNS} = slope \cdot DP + intercept \quad (6)$$

$$[redox]_{app} = [carbohydrate] \cdot rf_{DNS} \quad (7)$$

The slope and intercept refer to the values given by the equation in the graph in Figure 7. When the absorbance was plotted against $[redox]_{app}$ (Figure 10), all carbohydrates except glucose closely followed the same line and the small differences were found to be random. This was the expected result for the graph in Figure 6 and is an indication that this model accurately describes the chemistry behind the DNS reaction. The negative intercepts could be caused by a fixed amount of analyte degradation caused by dissolved compounds in the sample.

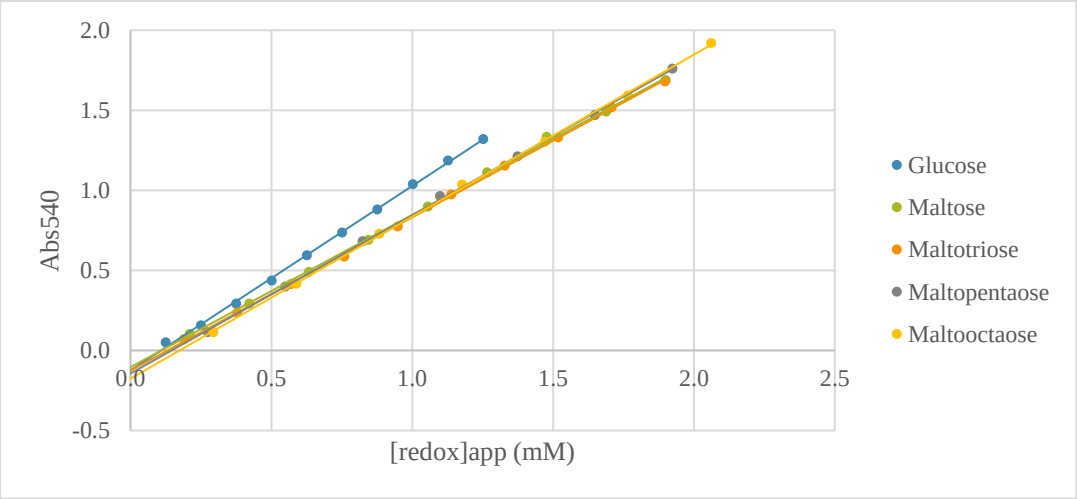


Figure 10. Absorbance plotted against the apparent concentration of reducing units of carbohydrates. Except for glucose, all followed close to the same line.

A possible explanation as to why the value of the slope of the graph in Figure 7 was higher than that of the intercept is that, while the C1 aldehyde can only be oxidized once before forming a carboxyl group, the C6 hydroxyl can be oxidized twice, i.e. first to and aldehyde and then to a carboxylic acid. Therefore, the C6 hydroxyl has the potential to reduce more DNS molecules and generate a stronger response. The slope value in this study was approximately 1.5 times greater than that of the intercept, which would mean that not all C6 hydroxyls were fully oxidized to carboxylic acids under the conditions used here. An alternative explanation would be that more hydroxyl groups than C6 were oxidised than just the C6.

The hypothesis derived from the results found in this study could be further strengthened by performing DNS analysis of glucans such as isomaltose, isomaltotriose and trehalose (Figure 11). The first two are di- and trisaccharides linked through α-1,6-glycosidic bonds and consequently have one C1 aldehyde and C6 hydroxyl each like a glucose molecule. They should therefore generate the same response. Trehalose is a disaccharide with an α-1,1-glycosidic bond. It lacks a reducing end but has two C6 hydroxyls, which means it should generate a response in between that of glucose and maltose. Further verification could be done with NMR by comparing the prevalence of hydroxyl, aldehyde and carboxyl groups in different samples before and after DNS reaction.

Considering that the results suggest the DNS method to be far less straight-forward than commonly believed, it was not used for characterization of the enzyme reaction products.

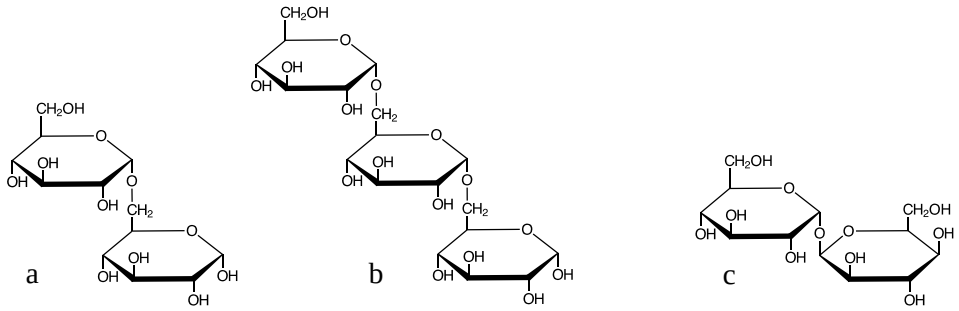


Figure 11. Molecular structures of glucans that could be used to further strengthen the hypothesis that the C6 hydroxyl contributes to the reducing ability of a glucan in the DNS assay. and with more reducing ability than the C1 aldehyde. The molecules are a) isomaltose. b) isomaltotriose and c) trehalose. Isomaltose and isomaltotriose are linked through α-1,6-glycosidic bonds. therefore having one C1 aldehyde and one C6 hydroxyl each like a glucose molecule. In trehalose. the two glucose repeating units are linked through an α-1.1-glycosidic bond. meaning there is no reducing end but two C6 hydroxyls.

4.1.4. Calculating the Amount of Branch Points in Maltodextrins

Based on Figure 7, it could be possible to estimate the molar fraction of α-1,6 branch points in a maltodextrin or other branched material with the aid of any maltooligosaccharide standard curve, if the average DP of the sample was known. The necessary equation is derived as follows:

The number of redox reactions caused by a pure, linear maltooligosaccharide, n_{RL}, is calculated as in Equation 8. This equation takes into account the difference in reducing capacity of the reducing end and the C6 hydroxyls.

$$n_{RL} = s \cdot n + i \tag{8}$$

n is the DP of the maltooligosaccharide, s and i are the slope and the intercept of a graph such as the one in Figure 7 that is based on the calibration curves of multiple carbohydrates in a homologous series. The number of reducing units in a pure, branched maltodextrin, n_{RB}, with p α-1,6-branch points is calculated as in Equation 9, the equation is rearranged by using Equation 8.

$$n_{RB} = n_{RL} - s \cdot p = s \cdot n + i - s \cdot p = s(n - p) + i \tag{9}$$

The nominal concentration, c_{nom}, of a maltooligosaccharide with DP n, mass m_n and molar mass M_n is calculated as in Equation 10.

$$c_{nom} = \frac{\left(\frac{m_n}{M_n}\right)}{V} \tag{10}$$

The concentration of reducing units, c_{red}, in the solution is calculated as in Equation 11, the expression is rewritten by using Equation 9.

$$c_{red} = c_{nom} \cdot n_{RB} = c_{nom}(s(n - p) + i) \tag{11}$$

The concentration of glucose repeating units, c_G, is calculated as in Equation 12.

$$c_G = n \cdot c_{nom} \tag{12}$$

Equations 13 and 14 are derived by combining and rearranging Equations 11 and 12.

$$c_{red} = \frac{c_G}{n}(s(n - p) + i) = c_G\left(s - \frac{s \cdot p}{n} + \frac{i}{n}\right) \tag{13}$$

$$c_{red} = c_G\left(s + \frac{i - s \cdot p}{n}\right) \tag{14}$$

4.2. HPLC-MS

HPLC-MS was used for characterization of the surfactant and for surfactant analysis of R_{L+} samples.

4.2.1. HPLC-MS Analysis of Brij® L23

The surfactant used in the enzymatic reaction was Brij® L23; a polyoxyethylene (EO) lauryl ether with an average of 23 EO groups. HPLC analysis of Brij® L23 in R_{L+} samples 0h, 1min, 9h and 48h (see Table 1 for starting materials and Table 2 for sample details) showed that the product was not homogenous, but contained an array of species of both different fatty chain length and number of EO repeats as shown in Figure 12. MS analysis revealed the presence of species with EO repeats of DP 14-25 and fatty acid chain lengths of 10-14 (see Appendix 7.2.1 for a table of detected molar masses and Appendix 7.2.2 for heat maps).

The apparent concentration of surfactant decreased with time, which could have been the result of degradation. However, it was considered more likely that it was an effect of complex formation with CDs. A lower CD fraction was observed in R_{L+} compared to that of R_{L-}, which supported the hypothesis.

If quantification had been possible, the loss of Brij® L23 could have been compared to the loss of CD to generate a complex composition with a hypothetical Brij® L23:CD ratio. This ratio could have been used to asses whether complex formation was the reason for the decreased detection of CD and Brij® L23 in R_{L+}. However, this would have required samples that did not evaporate and standards of EO decyl, lauryl and myristyl ethers (DP 10, 12 and 14 respectively) with different numbers of EO repeats.

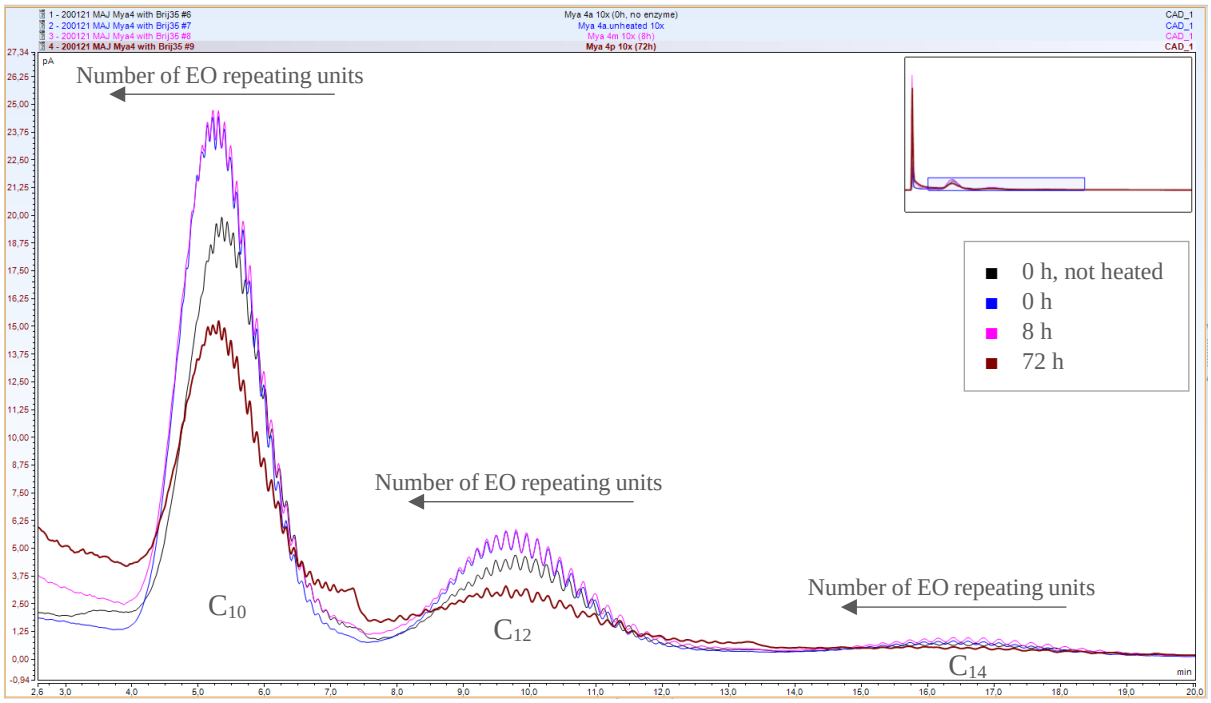


Figure 12. HPLC chromatogram of Brij® L23 in samples similar to those of R_{L+} that contained the long maltodextrin and the inert surfactant. Unlike the R_{L+} samples. the enzymatic inactivation was only carried out for 2.5 h except for the 0h sample that was not heated at all. Species with a fatty chain of lengths 10. 12 and 14 were detected and within those frames. species of head group lengths 14-25. These results were used as no MS data was recorded of the R_{L+} samples.

4.3. HPAEC

HPAEC was used for analysis of glucan species after the enzymatic reactions. Through HPAEC, it was possible to calculate average DP, identify changes in the product distribution profiles and to follow the CD production over time.

4.3.1. Chromatogram

A typical chromatogram post-enzymatic reaction is shown in Figure 13. Compounds with DP >1 generated one main peak and at least one smaller peak, labelled "s" in the chromatogram (DP 2-8). The two peaks represent the two anomers (α and β) of a given linear oligoglucan. The area of the main and "s" peaks were added together to calculate the amount of the given compound. Due to the increasing number of smaller peaks, the "s" notation was dropped after DP 8. These larger compounds were processed as timed groups instead of named groups.

The largest glucan identified had DP 42 and was present in the 0h sample of reaction R_{L-}. It is likely that even larger species were present, but due to their poor response, they were undetected. Had the concentrations of the larger glucans been higher, it is possible that they would have been identified too.

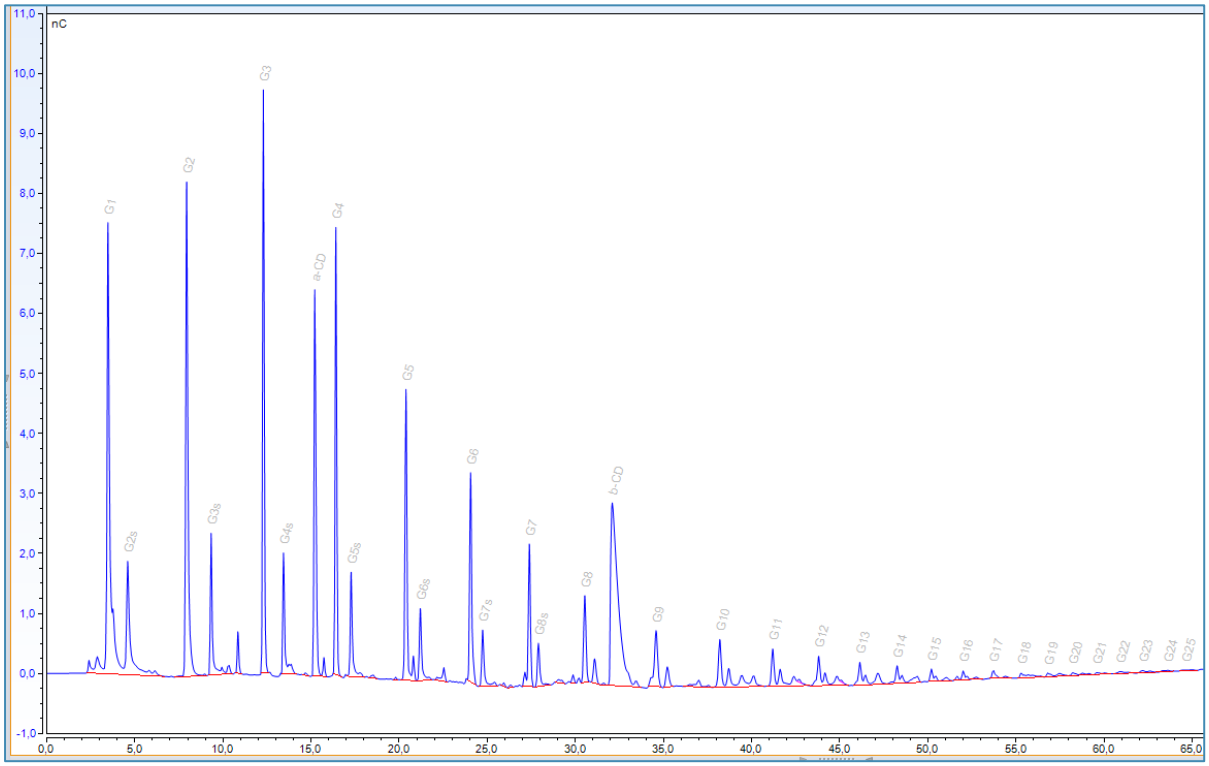


Figure 13. Typical chromatogram post enzymatic reaction.

4.3.2. Extrapolation of Calibration Curves

Digestion of glucans by CGTase resulted in an array of glucan species. Calibration curves were made for maltooligosaccharides of DP 1-8 and for α- and β-CD. As the response decreased for longer species, the results from the standard curves were used to extrapolate and estimate the response factors (rf_{HPAEC}) for longer maltooligosaccharides. The best-fitting curve type was the power trend line, in the form of Equation 15 and shown in Figure 14. a and b are constants, the experimental values and the R^2 values are presented in Appendix 7.3, as is the schematic of which standard curve was used to which samples. The unit of the response factor was nC·min/µg,mL and was used to calculate the concentrations of the longer glucan species as in Equation 16.

$$\mathrm{rf_{HPAEC}} = a \cdot \mathrm{DP}^{b} \tag{15}$$

$$[\mathrm{glucan}] = \frac{\mathrm{area}}{\mathrm{rf_{HPAEC}}} \tag{16}$$

[glucan] is the concentration of the glucan and the area refers to the integrated peak area found in the chromatogram assigned to the specific DP.

Due to the large number of samples, lengthy run times and the eluent limitations of the HPAEC, four different eluent batches were used. A standard curve was made for each batch, see Figure 14.

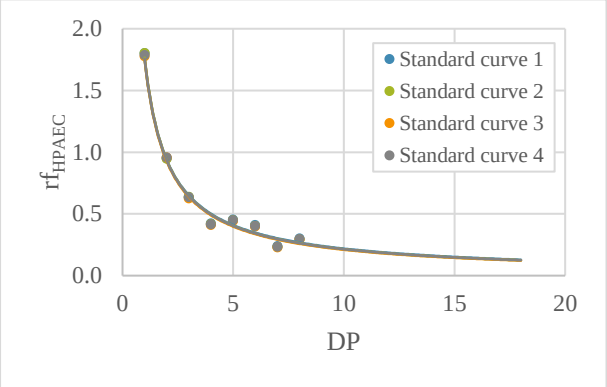


Figure 14. Graph of how the rf_{HPAEC} decreased for longer oligosaccharide species. Due to the large number of samples, four different eluent preparations and therefore also standard curves were used throughout the analysis. Extrapolation was based on calibration curves of species with DP of 1-8. The best fitting trend line was a power function in the form: $rf_{HPAEC} = a \cdot DP^b$. *a* and *b* are constants.

4.3.3. Calculation of Average DP

The average molar weight of the free linear sugar distribution, M_{av}, was calculated according to Equation 17 and the average DP according to Equation 18.

$$M_{av} = \frac{\sum m_i}{\sum n_i} = \frac{\sum n_i \cdot M_i}{\sum n_i} \tag{17}$$

$$\mathrm{DP} = \frac{M_{av} - M_{H_2O}}{M_{C_6O_5H_{10}}} = \frac{M_{av} - 18.0148}{162.1400} \tag{18}$$

m_i was the detected mass of non-cyclic fragments of DP i, n_i was the amount of non-cyclic fragments of DP i measured in moles and M_i was the assumed molar weights calculated as in Equation 19.

$$M_i = i \cdot 162.1400 + 18.0148 \tag{19}$$

4.3.4. Enzymatic Inactivation

After samples were collected from the reactors, the enzyme was inactivated to preserve the product profile as much as possible as it would otherwise have been altered by the continued enzymatic digestion. As the thermostable CGTase was inactivated through heating, stability tests were performed to assess the inactivation.

Inactivation at 95°C for 2.5 h proved to be unsuccessful as signs of enzymatic activity was detected in all four reactors. This is apparent in the chromatogram shown in Appendix 7.1, where multiple analyses of the same sample revealed that the product profile did not remain constant. The inactivation time was increased to >16 h. which led to a successfully inactivated enzyme. While the profiles were not fully preserved over the ten day course of the studies, this was deemed a consequence of using different calibration curves in combination with a small but negligible amount of residual or regained enzymatic activity.

The longer deactivation time in the shaking tray of the Thermo Shaker caused the lids of most sample vials to loosen. This caused partial or, as in the case of the R_{L+} 2h sample, full evaporation. This made calculations of absolute values futile and comparisons of the free sugar distributions were instead based on molar fractions. To avoid this issue in future studies, the lids could be covered with parafilm, or else the inactivation could be performed in Eppendorf tubes with caps instead of lids.

4.3.5. Enzymatic Reaction Results

The effect different glucan substrates had on the product distribution after enzymatic digestion was tested by using three different substrates. In addition, the effect of a surfactant was examined by comparing the results of reactions R_{L-} and R_{L+}, where the same maltodextrin was used as glucan substrate but R_{L+} also contained the inert surfactant Brij® L23. The surfactant cannot be elongated by the enzyme.

As partial amounts of the samples evaporated during the enzymatic inactivation, absolute amounts could not be used for comparisons. It was therefore not possible to assess whether the expected amount of sugar was found and thereby how good the response factor model was, or the instrument and process methods used for identifying compounds.

To gain a more detailed understanding of the initial changes, the enzyme concentration should either have been lower or samples should have been taken in closer intervals. An advantage of using a lower enzyme concentration would have been that, if there was some reactivation of the enzyme before all samples were analysed, this would have had a smaller impact on the sample compositions.

While all four reactions exhibited an apparent dramatic initial change in product composition, the DP and product profiles detected did not accurately reflect the sample composition at the time of sampling as the enzyme was proven not to be fully inactivated within 2.5 h. Rather, the results reflected the composition after the combination of the time in the reactor at 65 °C and of the unknown inactivation time at 95 °C. Other methods of enzymatic inactivation should be considered for future studies.

4.3.5.1. DP over Time

The change in average DP of the linear fraction of each reaction is shown graphically in Figure 15.

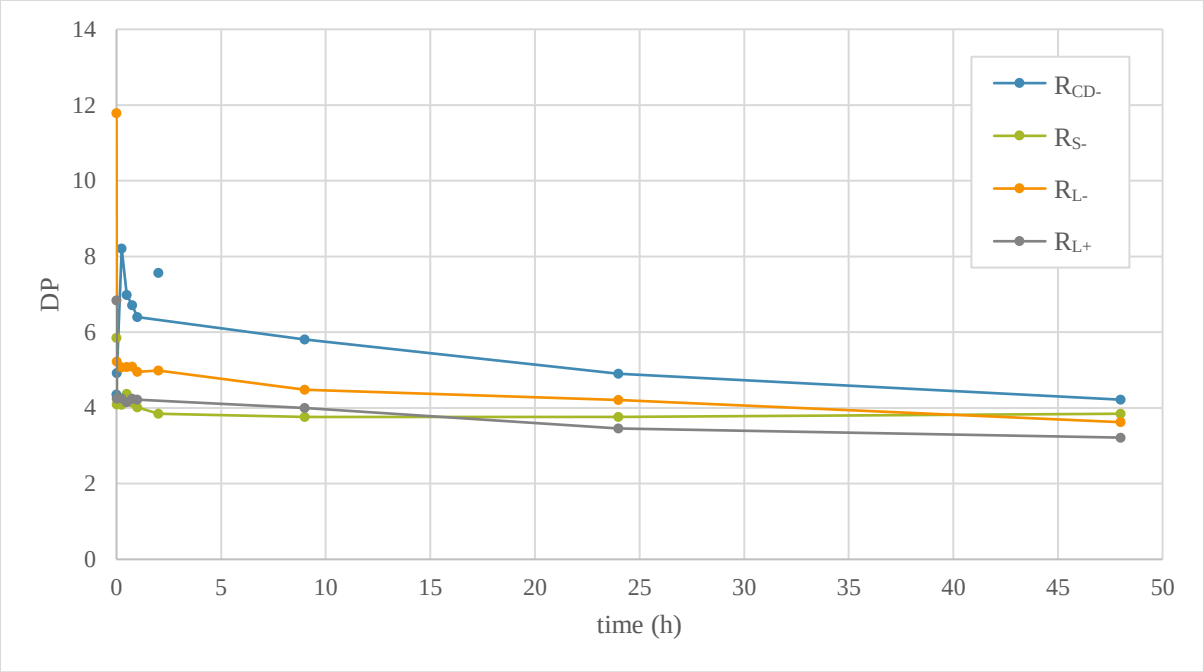


Figure 15. Graph over how the average DP within the linear fractions in the four enzymatic reactions changed over time. Samples A and G of R_{CD-} were removed from the graph. At the time of collection of the 0h sample. the reactor only contained α-CD and no enzyme. While trace amounts of linear carbohydrates were detected. these were not relevant to the graph. The 2h sample is an outlier and possibly caused by a mix-up of samples.

As expected, a decrease of DP over time was observed in all four reactions. The only exception is the DP of R_{CD-} which increased initially. This is due to the fact that no linear fragments were present at the start but were synthesized as soon as the enzyme was added to the reactor. The DP maximum seemed to have been reached at some point between the 0h and 15min samples. As there was an abundance of α-CD, the coupling reaction was likely the dominant reaction in the beginning. Opened CDs were linked onto each other, creating long chains before the other reactions took over.

The DP of R_{S-} did not change much after the first measurements and, in contrast to the DP of the other reactions, remained constant instead of decreasing throughout the 48 h. A possible explanation for the stabilized DP is that while the enzyme remained active in the reactor, the viable substrates were limited to such an extent that the linear fraction remained unchanged. The linear compounds were too short to be used as donor substrates and, while the CDs could still be used as donor substrates, they were likely mostly cleaved and reformed due to the high cyclization activity of the enzyme. The DP of R_{L-} and R_{L+} would likely have stabilized as well, had the reaction been allowed to continue.

Furthermore, the DP of the R_{L-} and R_{L+} reactions decreased to lower values than that of where the DP of R_{S-} stabilized. This could, however, be explained by the different origins of the shorter and longer maltodextrins. The shorter maltodextrin used in R_{S-} was sourced from potatoes and would likely be phosphorylated to some extent which would sterically hinder the enzymatic activity. In contrast, the longer maltodextrin used in R_{L-} and R_{L+} was sourced from maize. This difference would ultimately have led to a difference in achievable yield.

4.3.5.2. Molar Fractions over Time

Common for all reactions was that, just as was observed regarding the average DP, the apparent initial activity was very high, particularly the cyclization activity. The reactions all reached approximately the same composition after 48 h, namely that of 70-80 mol% linear sugars and 20-30 mol% CDs.

As seen in Figure 16, α-CD in R_{CD-} was initially degraded at a fast rate, creating equal amounts of β-CD and linear glucans in the first 2 h. After an increase in β-CD production at 9 h, the molar fraction of linear glucans grew steadily while both the β-CD and total CD fractions slowly decreased. The production of β-CD was higher but with a delayed peak compared to the reactions in which maltodextrins were used as substrates, likely due to the very different α:β CD ratio.

The initial enzymatic activity was high in R_{S-} (Figure 17), but the distribution between the molar fractions showed little change going forward. Compared to the other reactions, the initial change was less dramatic.

Within the first minute of R_{L-} (Figure 18), the CD molar fraction increased to more than 50%, after which it slowly decreased according to the same pattern as R_{CD-} and R_{L+}.

The apparent molar fraction of CDs in R_{L+} (Figure 19) was initially much lower than that of R_{L-} despite them having the same glucan substrate. A possible explanation is that the CDs formed inclusion complexes with the surfactant. This would have lowered the apparent CD concentration, as the CDs involved in complexes may not have been accessible for analysis. This theory was supported by the appearance of a white precipitate in the sample vials, presumably consisting of poorly soluble complex between CD and surfactant.

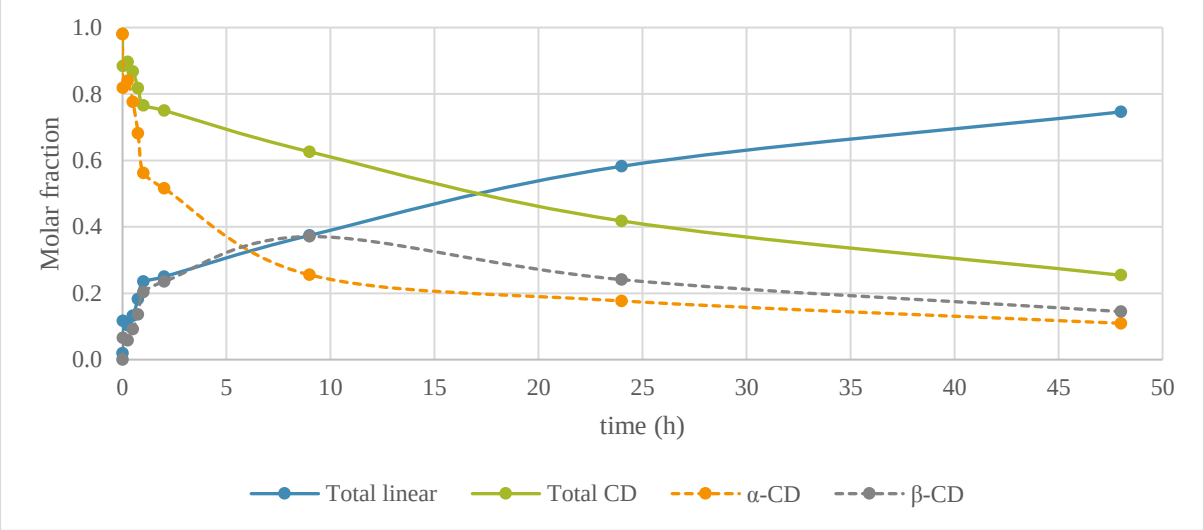


Figure 16. The R_{CD-} molar fractions of linear species, total CD fraction, α-CD and β-CD plotted against time.

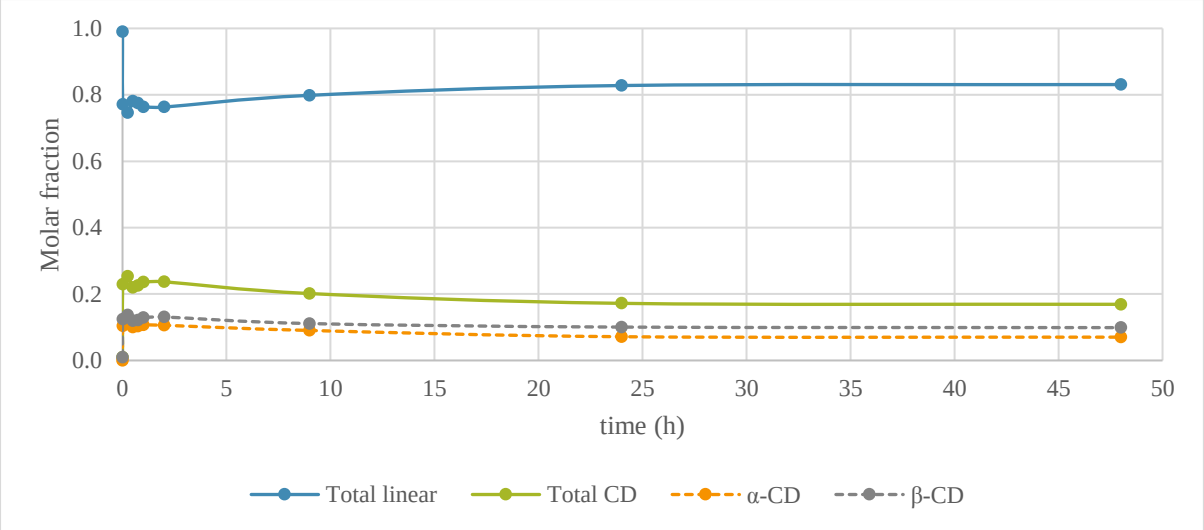


Figure 17. The R_{S-} molar fractions of linear species, total CD fraction, α-CD and β-CD plotted against time.

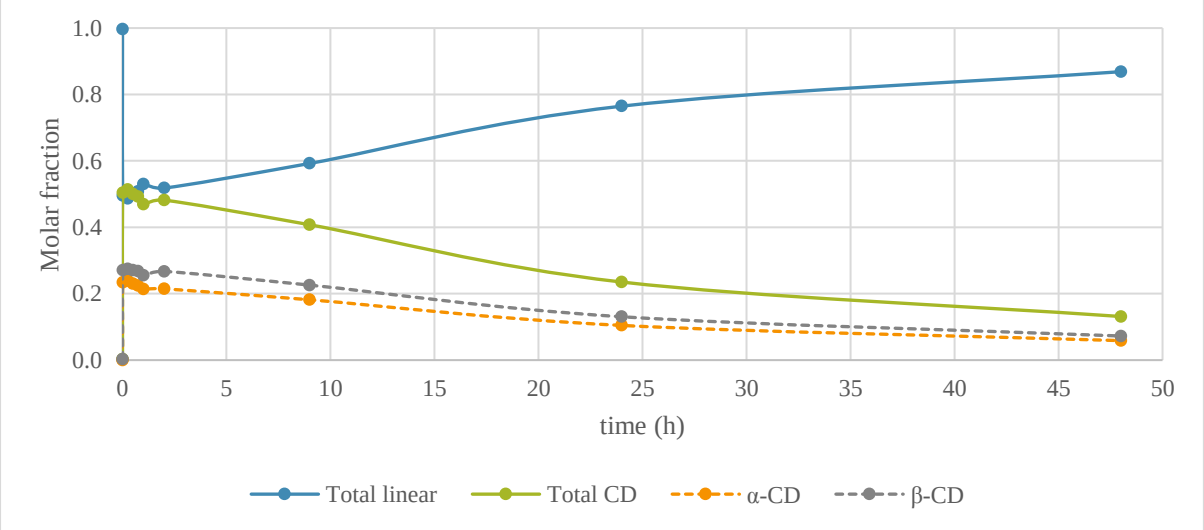


Figure 18. The R_{L-} molar fractions of linear species, total CD fraction, α-CD and β-CD plotted against time.

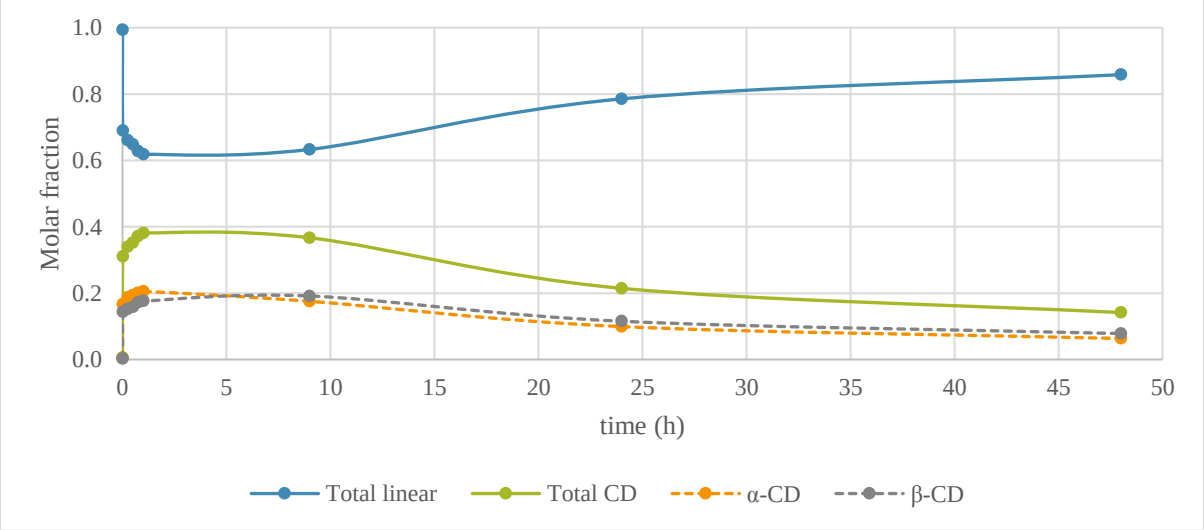


Figure 19. The R_{L+} molar fractions of linear species, total CD fraction, α-CD and β-CD plotted against time.

4.3.5.3. Substrate Effect

The most noticeable difference between using α-CD and maltodextrins as glucan substrate was the DP profile. As there was no linear fraction initially in R_{CD-}, it had a theoretical DP of 0. While the initial increase in DP was inevitable as the linear fraction was formed, the DP reached a surprisingly high number and remained distinctly higher than that of the other reactions. Due to a too low sampling frequency and inefficient enzymatic inactivation, the maximum DP was in all probability not detected.

The use of a shorter maltodextrin in R_{S-} compared to a longer in R_{L-} greatly reduced the achievable yield of CDs and species of higher DP, as the DP started just above the limit of what can be utilized as donor substrate. It should, however, be noted that the different origins of the substrates likely affected the outcome and it is not possible to know what effect can be attributed to what parameter.

4.3.5.4. Surfactant Effect

The presence of a surfactant in R_{L+} affected both the apparent CD molar fraction and the apparent DP. The apparent starting DP of R_{L+} was almost half of that in R_{L-}, despite them sharing the same glucan starting material. This fact could be explained by formation of inclusion complexes between the longer glucans and the surfactants. If the dissociation constant was low, that would mean a stable complex and could possibly explain why the DP of R_{L+} remained lower than that of R_{L-}.

A white precipitate appeared in the 0h sample of R_{L-} and in all samples of R_{L+} after storage at 4°C. As no enzyme was present in the 0h samples, the most likely explanation was that the sugars aggregated and precipitated because of their length in R_{L-} and, in addition, formed complexes with the surfactant in R_{L+}. After enzymatic digestion in R_{L-}, the longer sugars were digested and no longer aggregated. Meanwhile, the surfactant formed complexes with the CDs in all R_{L+} samples, affecting the apparent molar fractions.

4.3.5.5. pH over Time

The pH was stable in R_{CD-}, R_{L-} and R_{L+}, and decreased significantly in R_{S-} between the 9h and 24h samples. In R_{CD-}, the pH remained at 8-9 for the entire 48 h. The pH of R_{L-} and R_{L+} was stable at 6 and only decreased slightly in the 48h sample of R_{L-} to 5-6. The pH of R_{S-} was 8-9 in the 0h - 9h samples, after which the pH decreased to 5-6 in the 24h and 48h samples. The underlying reason for the decrease in pH is unknown.

4.3.5.6. Flory Distribution

The molar fractions of each sample were logarithmized and plotted versus the DP in order to elucidate if the linear fraction was distributed according to Flory statistics. Most R^2 coefficients (Appendix 7.4) of the 45min and succeeding samples were above 0.96, and the distribution of the 1h samples were the closest to linear with R^2 coefficients above 0.97 (Figure 20). While not perfectly linear, it was considered close enough to conclude that the free linear oligosaccharide fraction followed Flory distribution.

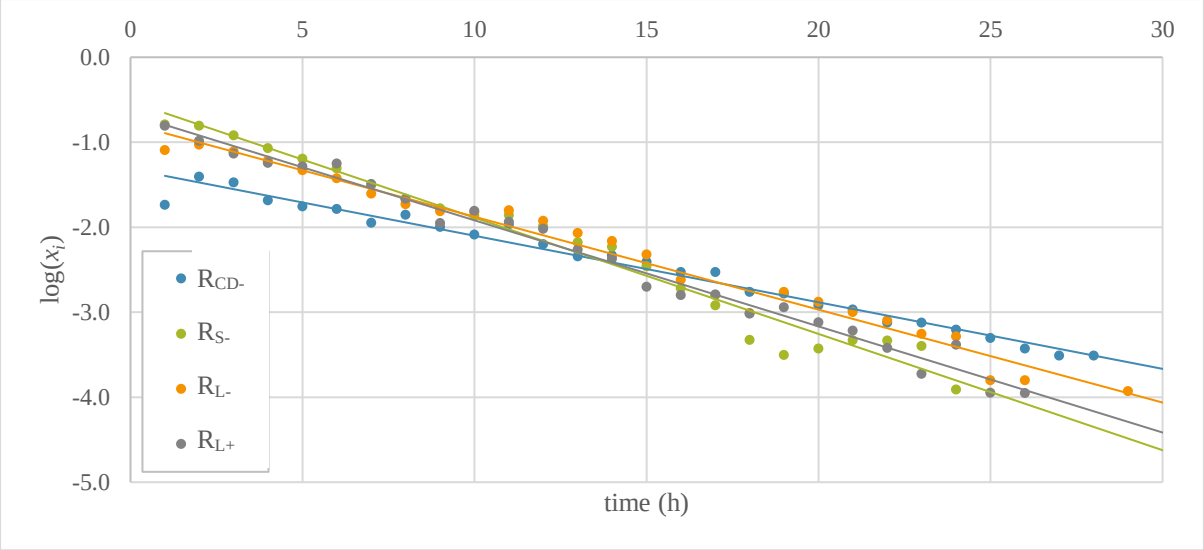


Figure 20. The linear relation between the logarithmized molar fractions and DP show that the free sugar fraction was distributed according to Flory in all four reactions. Data from 1h sample.

5. Conclusion and Future Studies

5.1. DNS

Strong indications were found that the increased reduction of DNS caused by carbohydrates of higher DP was caused by oxidation of groups other than the C1 aldehyde, likely the C6 hydroxyl group. This result should be further verified by replicating the experiments with a new batch of reagent solution.

Analysis of other glucans bound by non-α-1,4-glycosidic bonds could be used as a means of verifying the conclusions. Isomaltose and isomaltotriose are bound by α-1.6-glycosidic bonds, hence they only have one C1 aldehyde and one C6 hydroxyl each. They could be verified to give the same response as each other, and as glucose. Trehalose, which consists of two glucose units bound by an α-1,1-glcosidic bond, holds two free C6 hydroxyl groups. This substance could be used to see if the response would indeed be between that of glucose and maltose, as if some of the C6 hydroxyl are in fact oxidized more than one step, trehalose would be more reducing than glucose.

An equation to calculate branch points based on the known DP, the DNS result and a standard curve of a maltooligosaccharide (or glucose) was derived. The effectiveness of the equation could be verified by analysing a maltodextrin with NMR to estimate the amount of branch points and compare the NMR and DNS results. A comparison between the DNS results of maltose, isomaltose and isomaltotriose could also be a way of evaluating the equation.

As the DNS method has been assumed to only detect reducing ends, it is highly likely that it has been used incorrectly in previous studies. Unless the results from this study are specific to the protocol used, conclusions from some previous studies may have to be questioned. Quantitative studies that have not prepared a calibration curve for each compound and used this method for detection of that specific compound are likely to have drawn incorrect conclusions. Qualitative studies, on the other hand, where the method has been used for detection of an analyte or of a difference in total reducing activity should have reliable conclusions, assuming the study was well executed.

5.2. The Enzymatic Reaction

Regardless of the glucan substrate, all reactions proceeded toward a product composition of an average DP below 5 and with the majority of the free carbohydrates in non-cyclic form.

Using α-CD as glucan substrate, rather than using a maltodextrin, resulted in a higher average DP within the linear fraction, particularly within the first hours. In this reaction, R_{CD-}, there was also a higher CD fraction and a distinctly different β-CD profile throughout the full testing time. The high initial CD levels likely promoted a higher coupling activity and an enhanced elongation of fragments, explaining the higher average DP.

Unlike the reaction where a longer maltodextrin was used as substrate, R_{L-}, the reaction where a shorter was used, R_{S-}, showed only faint traces of enzymatic activity throughout the 48 hours. This was probably due to most glucan material having been digested to fragments with too low DP to allow them to be viable substrates. The enzyme was thought to still be active, but with low net change of the product profile. The R_{S-} reaction stabilized at a higher DP than both R_{L-} and R_{L+} reactions. This could have been due to phosphorylation or branching of the potato-derived maltodextrin used in R_{S-}, thereby limiting the viable fraction of the substrate. This theory could be a point for future studies.

The presence of the inert surfactant caused the apparent CD fraction to be distinctly lower. A likely explanation is the formation of inclusion complexes; CDs trapped in complexes with the surfactants may not be available for detection, which in turn would affect the apparent molar fractions.

Regardless of the glucan substrate, the free linear sugar fractions were distributed according to Flory statistics.

A high initial activity was observed in all enzymatic reactions, regardless of glucan substrate used. However, as the enzyme was proven to be active for another few hours during enzymatic inactivation, it is impossible to know how accurately the results represented the product profile at the time of sampling. If the study was to be continued, alternative methods for enzymatic inactivation should be evaluated. The presence of inclusion complexes should be verified to explain the apparent loss of longer linear oligosaccharides, Brij® L23 and CDs. The analysis method should be improved to allow for detection of γ-CD. Although γ-CD likely constituted a small molar fraction, the results would be more accurate had the γ-CD fraction been included in the calculations.

Finally, it would be of interest to further explore how the product profile is affected by different kinds of substrates, e.g. different mixes of linear and cyclic glucans, or by continuous addition substrate.

6. References

References/Bibliography

BENDER, H. 1990. Studies of the mechanism of the cyclisation reaction catalysed by the wildtype and a truncated α-cyclodextrin glycosyltransferase from Klebsiella pneumoniae strain M 5 al, and the β-cyclodextrin glycosyltransferase from Bacillus circulans strain 8. *Carbohydrate research,* 206**,** 257-267.

DAVIES, G. J., WILSON, K. S. & HENRISSAT, B. 1997. Nomenclature for sugar-binding subsites in glycosyl hydrolases. *Biochemical Journal,* 321**,** 557.

FLORY, P. J. 1936. Molecular size distribution in linear condensation polymers1. *Journal of the American Chemical Society,* 58**,** 1877-1885.

HÜBNER, H. 1879. Ueber Nitrosalicylsäuren und die Isomerieen der Benzolabkömmlinge. *Justus Liebigs Annalen der Chemie,* 195**,** 1-55.

KLEIN, C., HOLLENDER, J., BENDER, H. & SCHULZ, G. E. 1992. Catalytic center of cyclodextrin glycosyltransferase derived from x-ray structure analysis combined with site-directed mutagenesis. *Biochemistry,* 31**,** 8740-8746.

MCCARTER, J. D. & WITHERS, G. S. 1994. Mechanisms of enzymatic glycoside hydrolysis. *Current opinion in structural biology,* 4**,** 885-892.

MERCK. *Enzymatic Assay of β-AMYLASE (EC 3.2.1.2)* [Online]. Available: https://www.sigmaaldrich.com/technical-documents/protocols/biology/enzymatic-assay-of-b-amylase.html [Accessed 2020-03-10 n.d.].

MILLER, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical chemistry,* 31**,** 426-428.

MOSI, R., HE, S., UITDEHAAG, J., DIJKSTRA, B. W. & WITHERS, S. G. 1997. Trapping and characterization of the reaction intermediate in cyclodextrin glycosyltransferase by use of activated substrates and a mutant enzyme. *Biochemistry,* 36**,** 9927-9934.

PENNINGA, D., STROKOPYTOV, B., ROZEBOOM, H. J., LAWSON, C. L., DIJKSTRA, B. W., BERGSMA, J. & DIJKHUIZEN, L. 1995. Site-directed mutations in tyrosine 195 of cyclodextrin glycosyltransferase from Bacillus circulans strain 251 affect activity and product specificity. *Biochemistry,* 34**,** 3368-3376.

ROHRER, J. 2013. Analysis of Carbohydrates by High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAE-PAD). Thermo Fisher Scientific Inc.

ROSEN, M. & KUNJAPPU, J. 2012. *Surfactants and Interfacial Phenomena*.

SAQIB, A. A. N. & WHITNEY, P. J. 2011. Differential behaviour of the dinitrosalicylic acid (DNS) reagent towards mono-and di-saccharide sugars. *Biomass and bioenergy,* 35**,** 4748-4750.

STROKOPYTOV, B., PENNINGA, D., ROZEBOOM, H. J., KALK, K. H., DIJKHUIZEN, L. & DIJKSTRA, B. W. 1995. X-ray structure of cyclodextrin glycosyltransferase complexed with acarbose. Implications for the catalytic mechanism of glycosidases. *Biochemistry,* 34**,** 2234-2240.

SUMNER, J. B. & GRAHAM, V. 1921. Dinitrosalicylic acid: a reagent for the estimation of sugar in normal and diabetic urine. *J Biol Chem,* 47**,** 5-9.

TERADA, Y., YANASE, M., TAKATA, H., TAKAHA, T. & OKADA, S. 1997. Cyclodextrins Are Not the Major Cyclic a-1,4-Glucans Produced by the Initial Action of Cyclodextrin Glucanotransferase on Amylose. *The Journal of Biological Chemistry,* 272**,** 15729-15733.

UITDEHAAG, J., VAN DER VEEN, B. A., DIJKHUIZEN, L. & DIJKSTRA, B. W. 2002. Catalytic mechanism and product specificity of cyclodextrin glycosyltransferase, a prototypical transglycosylase from the α-amylase family. *Enzyme and Microbial Technology,* 30**,** 295-304.

7. Appendix

7.1. 2.5 h Ineffective Enzyme Inactivation

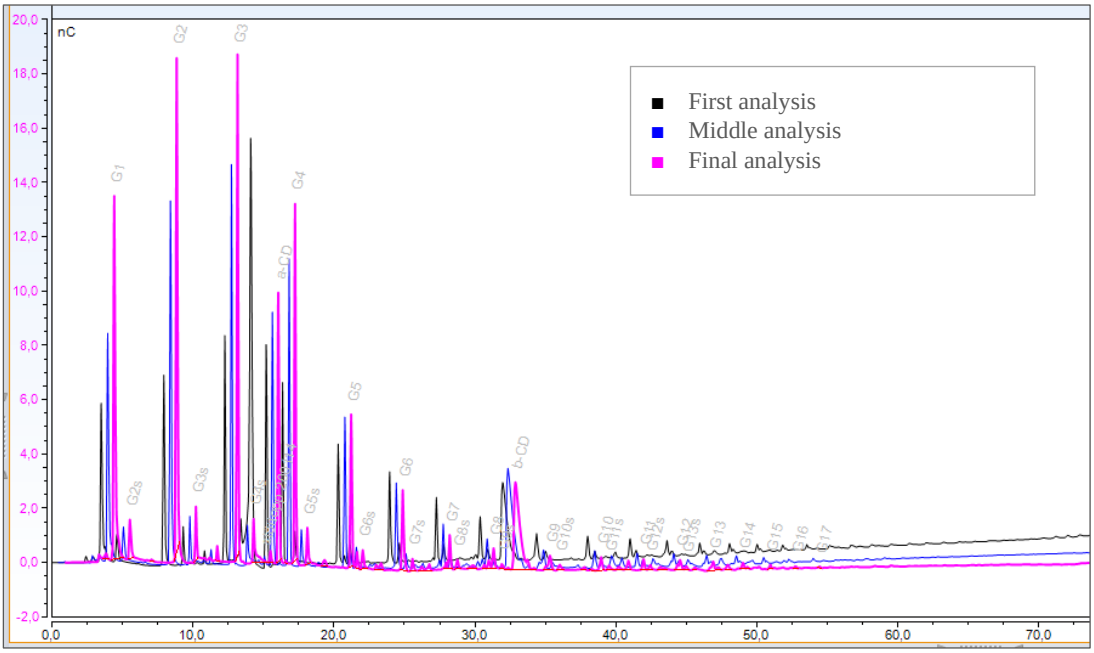


Figure 21. Chromatograms with time offset of the same sample at three different times over ten days. The change in product profile with an increase in shorter species and α-CD and the decrease of longer species showed that the enzyme was not properly inactivated after heat treatment for 2.5 h.

7.2. MS Raw Data

Unfortunately, no MS data was collected during the analysis of R_{L+}. The MS data below was collected after the reaction that was only inactivated for 2.5 h.

7.2.1. Detected Molar Masses

Molar masses identified in BRIJ ® L23.The values within parentheses where not found and the value in bold, 1196.8, is the molar mass of the expected surfactant.

	C10	C12	C14
E14	772.5	800.6	828.6
E15	816.6	844.6	872.6
E16	860.6	888.6	916.7
E17	904.6	932.7	960.7
E18	948.6	976.7	1004.7
E19	992.7	1020.7	1048.7
E20	1036.7	1064.7	1092.8
E21	1080.7	1108.8	1136.8
E22	1124.8	1152.8	1180.8
E23	1168.8	**1196.8**	1224.8
E24	1212.8	1240.8	(1268.9)
E25	1256.8	(1284.9)	(1312.9)

7.2.2. MS Heat Maps

MS heat maps for R_{L+} samples 0h. 8h heated and 72h. Additionally, one of the 0h samples had not been treated to the thermal inactivation for 2.5 hours.

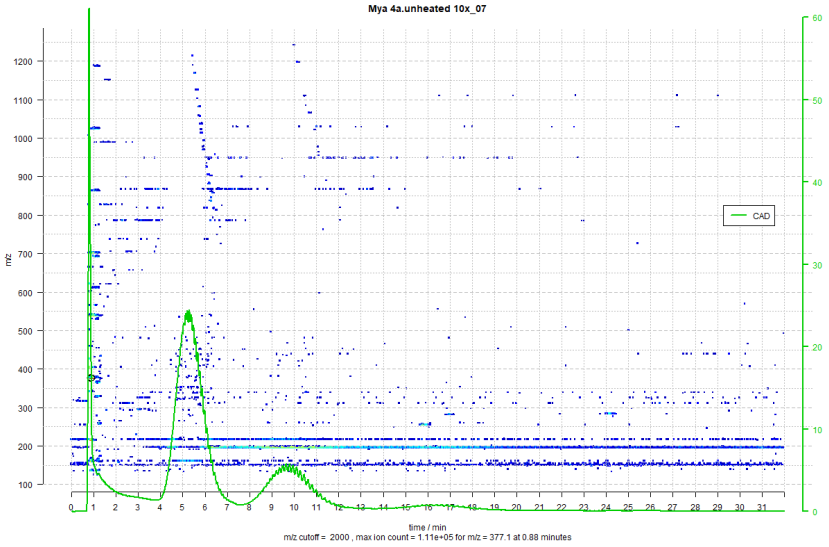


Figure 22. Heat map for R_{CD-} 0h sample without thermal inactivation.

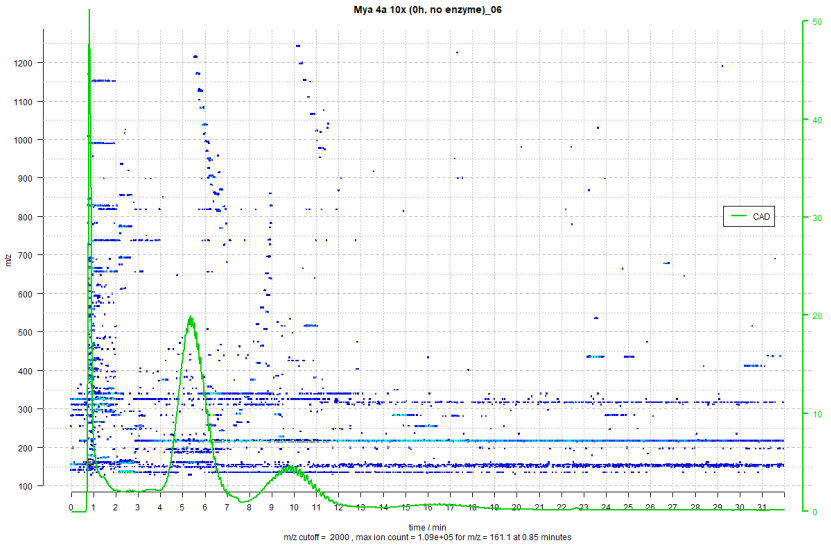


Figure 23. Heat map for R_{CD}- 0h sample after 2.5 h thermal inactivation.

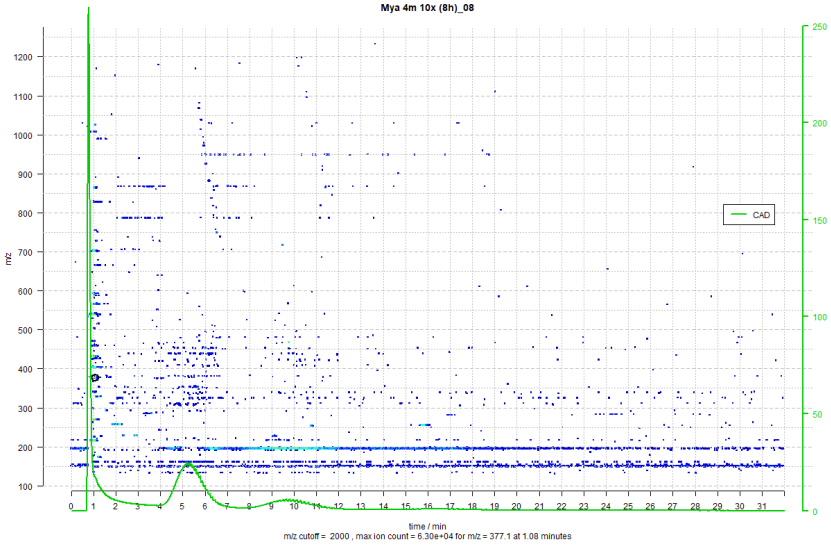


Figure 24. Heat map for R_{CD}- 8h sample after 2.5 h thermal inactivation.

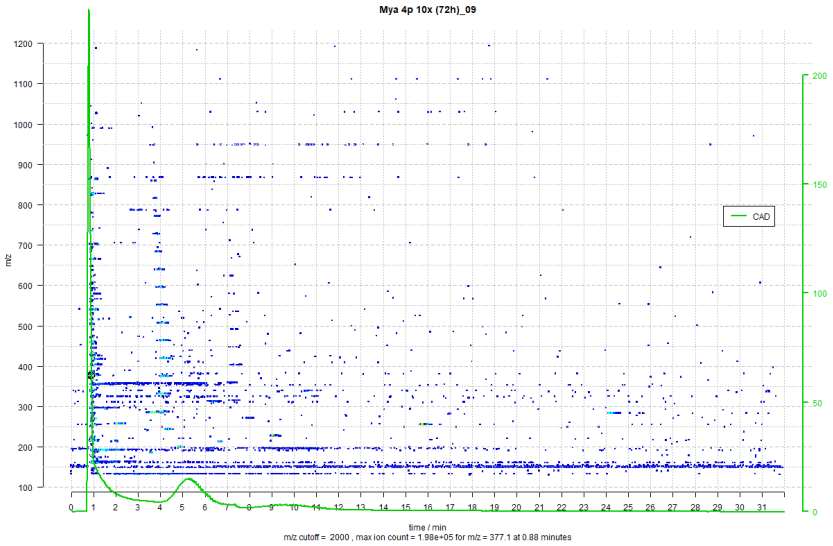


Figure 25. Heat map for R_{CD-} 72h sample after 2.5 h thermal inactivation.

7.3. HPAEC Standard curves

The experimental values of the equations of the trend lines in Figure 14. The equations were in the form $rf = a \cdot DP^b$.

	a	**b**	**R^2**
Standard curve 1	1.7566	-0.9055	0.9532
Standard curve 2	1.7649	-0.9177	0.9542
Standard curve 3	1.7532	-0.9210	0.9535
Standard curve 4	1.7580	-0.9143	0.9530

7.4. Flory Distribution R^2 Values

	R_{CD-}	**R_{S-}**	**R_{L-}**	**R_{L+}**
0h	0	0.975	0.833	0.975
1min	0.950	0.979	0.897	0.904
15min	0.923	0.982	0.980	0.971
30min	0.880	0.986	0.982	0.969
45min	0.965	0.978	0.980	0.986
1h	0.978	0.974	0.979	0.986
2h	0.968	0.943	0.982	-
9h	0.979	0.977	0.953	0.987
24h	0.984	0.963	0.977	0.981
48h	0.975	0.979	0.966	0.945

Popular Science Summary

Carbohydrate Analysis and how CGTase is Affected by Different Raw Materials

In this study, strong indications were found that the DNS method – a century old method for carbohydrate analysis – does *not* work as previously thought. The enzyme CGTase was also tested with different raw materials to gain a better understanding of how it works.

Until now, the DNS molecule has been though to only react with one specific part of certain sugars called *reducing sugars*. As the different kinds of reducing sugars all have one such part per molecule, this method has been used as an easy way of counting the total number of sugar molecules in a sample. In this study, however, more parts of the reducing sugar molecules reacted. This meant that larger molecules gave a higher signal. If this is general to all different ways of using the DNS method, then that would mean that the method has often been used incorrectly, and that conclusions from previous studies may be wrong.

In the second part of the study, it was investigated how the enzyme CGTase acts when it has different raw materials. The enzyme has the ability to catalyse four different reactions, which makes it possible to generate very different products. In general, the product profiles were similar, but a few observations were made. The enzyme produced longer products when it had circular sugars called α-cyclodextrins as raw material, and shorter products with short, straight sugar chains called maltodextrins. A general trend was found that, no matter what the raw material was, the end products followed a statistical distribution model that is sometimes seen in certain polymerisation reactions. It is important to understand the enzyme you are working with, as that makes it possible to give the enzyme the best possible opportunity to do its job.

The analysis of the enzyme's products was done with HPAEC, which is a type of liquid chromatography designed specifically for carbohydrate analysis.