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TEMPO oxidation of gelatinized potato starch results in acid resistant blocks of glucuronic acid moieties

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ABSTRACT

Chemical derivatization is often applied to improve polysaccharide functionality. Primary hydroxyl groups in starch can, for example, be oxidized to aldehydes by using a 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO)-mediated reaction. The major part of the aldehydo groups is subsequently converted to carboxyl groups by NaOCl. The exact structure of TEMPO-oxidized starch was studied to promote a better understanding of the TEMPO oxidation mechanism and the functionality of oxidized starches. By using weak and strong acidic hydrolysis, and methanolysis, at elevated temperatures, oxidized starches with different degrees of oxidation (DO) were broken down into oligomers and monomers. Analysis of the oligomers by chromatographic and mass spectrometric techniques revealed that blocks of glucuronic acid moieties are present in the oxidized starch polymers. The α(1 → 4) glucuronic acid–glucuronic acid bond was found to be very resistant to breakdown by acids. The α(1 → 4) glucuronic acid–glucose bond also showed increased resistance to acids compared to α(1 → 4) glucose–glucose bonds. The size of the blocks of glucuronic acid moieties increased when DO increased. Furthermore, the presence of clusters of aldehydes close to carboxyl groups directly after oxidation was proven. This implies that TEMPO, which is positively charged in its active state, is apparently attracted by the negatively charged carboxyl groups. Because of this, TEMPO tends to be active in areas where carboxyl groups have already been formed, which leads to a block wise distribution of the glucuronic acid moieties.

1. Introduction

Chemical and enzymatic derivatizations are commonly used to improve polysaccharide functionality. Starches can be oxidized using hypochlorite under alkaline conditions (Potze & Hiemstra, 1963; Taggart, 2004). These oxidized starches find various applications in the food industry because of their high stability, low viscosity, clarity and film-forming properties (Kuakpetoon & Wang, 2006).

Polysaccharides can also be oxidized by using a 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO)-mediated reaction. Hypochlorite and bromide are used as the regenerating agents in this case. One of the first published procedures for such an oxidation was applied on starch (De Nooy, Besemer, & Van Bekkum, 1995a). This reaction is more than 95% specific for the primary alcohol groups present in the starch polymer (De Nooy et al., 1995a). The TEMPO-mediated oxidation reaction was, furthermore, shown to have yields of almost 100% when reagents suffice (Ding et al., 2008). The yield and selectivity of the oxidation reaction were found to depend on the temperature and the concentration of NaBr and TEMPO (Suh, Chang, & Kim, 2002). Kato et al. performed a study on the formation of intermediate structures during the TEMPO oxidation process. They found that significant amounts of intermediate aldehydo groups at the C-6 position are likely to be formed during the TEMPO oxidation of water-soluble starch. These groups can form hemiacetals with water or with hydroxyl groups of the starch itself, and will be further oxidized into C-6 carboxyl groups if reagents and reaction time suffice (Kato, Matsuo, & Isogai, 2003).

TEMPO-oxidized polymers find their application in the non-food area. Carboxyl and carbonyl functionalities are known to be important in the pulping process and have an influence on paper properties (Bragd, Van Bekkum, & Besemer, 2004). Recently (Li et al., 2009), potato starches were TEMPO-oxidized to several degrees of oxidation (DO) and subsequently cross-linked into a micro gel using sodium trimetaphosphate. It appeared that the DO determined the cross-linking efficiency and could, therefore, be used to adjust the density of the gel. This was applied in order to make the most suitable gel for a specific controlled-release application.

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In this study, the structure of TEMPO-oxidized potato starches with different DO was elucidated through analysis of degradation products after weak and strong acidic hydrolysis and strong acidic methanolysis of the polymers. This provides insight into the distribution of the oxidized groups along the starch backbone and, therefore, also into the mechanism of TEMPO oxidation. Also the vulnerability of the different types of bonds present in the polymer to the breakdown by using acids will be discussed.

2. Experimental

2.1. Materials

Potato starch was obtained from AVEBE (Foxhol, The Netherlands). Sodium hypochlorite was from Breustedt Chemie (Apeldoorn, The Netherlands) and TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl) from Degussa (Darmstadt, Germany). Sodium bromide (99%), sodium borodeuteride (98%), trifluoroacetic acid (TFA), D-(+)-glucose (Glc), methyl α-D-glucopyranoside, and D-glucuronic acid (GlcA) were purchased at Sigma–Aldrich (St. Louis, MO, USA). HCl in methanol (3N) was obtained from Supelco (Sigma–Aldrich) and diluted to 2 M HCl in methanol by using methanol from a fresh bottle obtained from J.T. Baker (Phillipsburg, NJ, USA). Acetonitril came from Biosolve (Westford, MA, USA), ethanol from Nedalco (Bergen op Zoom, The Netherlands), and 2,5-dihydroxybenzoic acid (DHB) from Bruker Daltonics (Bremen, Germany).

2.2. Methods

2.2.1. Procedure for starch oxidation

For oxidation, the procedure used was based on work previously published (Besemer & De Nooy, 1995; De Nooy et al., 1995a). Twenty-five grams of potato starch (21 g dry matter) was suspended in 460 ml water and gelatinized. The solution was then kept below 8 °C. The solution was brought to pH 10 after which 100 mg of TEMPO and 100 mg of NaBr were added. A solution of NaOCl was then added continuously. During the oxidation process, the degree of oxidation (DO) was monitored by the amount of alkali that was added to keep the pH at 10. DO was defined as: average number of GlcA moieties present per 100 anhydrohexose moieties (Glc and GlcA together) in the starch polymer. When the desired DO had been reached, the influx of NaOCl was stopped and 20 ml of ethanol was added. Remaining aldehydo groups were reduced by introducing 200 mg of $NaBD_4$ and after 1 h the pH was set at 6.5 by using 4 M HCl. Products were then precipitated in 66% ethanol, washed, and then redissolved in water at pH 3, after which 20% methanol was added. Borate esters and remaining methanol were removed using a rotating film evaporator, and the remaining solution was freeze-dried. Starches with DO 30, 50, and 70 were obtained in this way. Products were stored at room temperature.

2.2.2. Starch hydrolysis using TFA

For starch hydrolysis, 2 ml of either 0.05 M or 2 M TFA in MilliQ water was added to 20 mg of starch sample in sealed glass incubation tubes. These samples were subsequently incubated for 2, 4, 8 or 20 h in a heating block at 100 °C. After cooling down, samples were dried using a stream of dry air. When samples were nearly dry, small amounts of methanol were added to assist the evaporation of TFA. Dried samples were stored at −20 °C until analysis.

2.2.3. Starch methanolysis using HCl

The procedure used for methanolysis is based on a procedure followed by De Ruiter, Schols, Voragen, and Rombouts (1992). Two milliliter of 2 M HCl in dry methanol was added to 5 mg of starch sample in glass incubation tubes. The remaining air in the tube was replaced by dry N_2 and the tubes were closed well to prevent drying out. These samples were subsequently incubated for 2, 4, 8 or 20 hours in a heating block at 80 °C. After cooling down, samples were dried using a stream of dry air and stored at −20 °C until analysis. Twenty percent of the sample volume of each of the 20-h incubated samples was transferred to a clean tube and dried, after which 1 ml of 2 M TFA in milliQ water was added. These tubes were sealed and subsequently incubated for 1 h at 121 °C in a heating block. Samples were then cooled down, dried under a stream of dry air, and stored at −20 °C until further analysis.

2.2.4. Determination of the molecular size distribution using HPSEC

Before and after incubation, the molecular size distribution of samples was determined by using High Performance Size Exclusion Chromatography (HPSEC). For this analysis, a Dionex Ultimate 3000 HPLC system (Dionex Corporation, CA, USA) was used. This system was equipped with three TosoHaas TSKgel SuperAW columns in series (4000–3000–2500, 150 mm × 6 mm) preceded by a SuperAW-L guard column (TosoH, Japan). After injection of 20 μl of a 2 mg/ml sample solution in 100 mM acetate buffer (pH 5.0), elution was performed with 0.2 M $NaNO_3$ at 40 °C, using a flow rate of 0.5 ml/min. A Shodex RI-101 RI detector (Showa Denko K.K., Japan) was used. A calibration curve was made by means of a series of pullulans with known molecular weights. Data were processed using Chromeleon software (Dionex Corporation).

2.2.5. Quantification of monomers and analysis of oligomers using HPAEC

After incubation, samples were analyzed using High Performance Anion Exchange Chromatography (HPAEC). A Dionex ICS-3000 HPLC system coupled to a Carbopac PA 1 Guard column (2 mm × 50 mm) and a Carbopac PA 1 column (2 mm × 250 mm) was used for this analysis (Dionex Corporation). Detection of the eluted compounds was performed by an ED40 EC-detector running in the Pulsed Amperometric Detection (PAD) mode.

To enable quantification of monosaccharides and to get a first impression of the sample composition, 10 μl of a 25 μg/ml sample solution in 100 mM Na-acetate buffer pH 5.0 was injected. After injection, compounds were eluted using a flow rate of 0.3 ml/min at 20 °C. Elution was started using a linear gradient from 0.05 M NaOAc in 0.1 M NaOH to 0.3 M NaOAc in 0.1 M NaOH in 20 min. This was followed by a linear gradient to 1 M NaOAc in 0.1 M NaOH in 20 min. After this, the column was washed for 10 min using 1 M NaOAc in 0.1 M NaOH and equilibrated for 15 min at 0.05 M NaOAc in 0.1 M NaOH. Calibration curves were obtained by injecting solutions of glucose, methyl α-D-glucopyranoside and glucuronic acid with concentrations varying from 1 to 25 μg/ml.

To get a better view on the less abundant fragments that were below the sensitivity level in the monomer analysis, and to improve the separation of the large and/or acidic oligomers, samples were injected a second time using different conditions. In this case, 10 μl of 2 mg/ml sample solutions in 100 mM Na-acetate buffer pH 5.0 were injected. Elution was again performed at a flow rate of 0.3 ml/min, 20 °C, but the gradient was changed. A linear gradient from 0.05 M NaOAc in 0.1 M NaOH to 0.4 M NaOAc in 0.1 M NaOH in 35 min was followed by a linear gradient to 1 M NaOAc in 0.1 M NaOH in 30 min. After this, the column was washed for 5 min using 1 M NaOAc in 0.1 M NaOH, followed by equilibration for 15 min at 0.05 M NaOAc in 0.1 M NaOH.

Data were processed using Chromeleon Software (Dionex Corporation).

2.2.6. Fragment structure analysis using MALDI-TOF MS

Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) was used to study the exact molecular weight of the fragments released during starch degradation. Prior to analysis, samples were diluted to a concentration of 0.5 mg/ml and subsequently desalted by adding a small spatula of Dowex AG50W-X8 Resin in the hydrogen form (Bio-Rad, Hercules, CA, USA) to 100 μl of sample solution. Dowex was then removed immediately by using Ultrafree-MC Durapore PVDF 0.22 μm centrifugal filter devices (Millipore, Bedford, MA, USA). The desalted sample (1 μl) was mixed on a target plate with 1 μl of matrix solution (10 mg DHB in 1 ml milliQ/ACN 70/30 (v/v)) and dried using a stream dry air.

Analysis was performed using an Ultraflex MALDI-TOF MS (Bruker Daltonics) equipped with a 337 nm nitrogen laser. The MS ran in the positive and reflector mode. After a delayed extraction time of 120 ns was applied, ions were accelerated using a 25 kV kinetic energy. The lowest laser intensity required to obtain an acceptable signal-to-noise ratio was used. Measurements were performed in the m/z 500–3000 range. The MS was calibrated using a mixture of maltodextrins (dextrose equivalent 20) with known molecular weights (in the appropriate range). Data were processed using flexAnalysis software (Bruker Daltonics). Based on the relative intensities observed for each oligomer, data were converted into bar graphs. The term "NGO" (Number of Glucuronic acid moieties per Oligomer) was used to indicate the number of glucuronic acid moieties present per oligomer. It was assumed that all molecules were equally ionizable.

2.2.7. Calculation of the number of deuterium atoms present in oligomers

The number of deuterium atoms resulting from aldehyde reduction by using $NaBD_4$, present in oligomers, was calculated based on isotopic distributions in MALDI-TOF mass spectra. The abundance of the natural ^{13}C isotope was assumed to be 1.109%. Furthermore, it was assumed that only non-oxidized Glc moieties could carry a deuterium and that this could only be one deuterium per monomer. The isotopic ratio was subsequently simulated for the several NGOs present, using a binomial distribution. Thereby a random distribution of deuterium in the oligomers was assumed that may be dependent on the DO. The average percentage of Glc carrying a deuterium atom in a certain type of oligomer in the sample followed then from the calculated isotopic ratio that showed the most optimal fit with the measured data. This best fit was based on least square calculations.

3. Results and discussion

Analyzable fragments had to be created to study the structure of oxidized potato starches. Starch samples with various degrees of oxidation (DO) were, therefore, hydrolyzed or methanolyzed and subsequently analyzed. The results will be discussed on the basis of the different analysis techniques used.

3.1. Molecular weight distribution of samples before and after acidic treatment

Non-oxidized starch and starches with DO 30, 50, and 70 were separately treated with 50 mM TFA, 2 M TFA, and 2 M HCl (in MeOH). Fig. 1 shows the HPSEC results obtained after analysis of the oxidized starches before and after a 20-h treatment with the acids mentioned.

The HPSEC profiles of the oxidized starches before acidic treatment (Fig. 1A) show that hydrodynamic volume distributions of the starches with DO30 and DO70 are similar, while the hydrodynamic volume of the starch molecules with DO50 is somewhat lower. Such behavior can be explained in two ways. On the one hand, starch molecules will be partly broken down during the oxidation process because of unstable intermediates (Kato et al., 2003), causing the hydrodynamic volume to decrease. On the other hand, more charged groups present may cause repulsion within the starch molecule and will, therefore, increase its hydrodynamic volume. In DO30 starch, both effects are relatively small, while the repulsion resulting from the high number of charged groups in DO70 starch compensates for the effect of starch breakdown. The lower number of unstable aldehydes expected in DO70 starch compared to lower DOs makes it, furthermore, less prone to degradation. Therefore, these starches have a similar apparent hydrodynamic volume distribution. In DO50 starch, the effect of breakdown on the hydrodynamic volume seems to be larger than the effect of charged groups. Therefore, these starch molecules have a lower apparent hydrodynamic volume.

After 50 mM TFA hydrolysis for 20 h, starches have been broken down partly to monomers, but a significant quantity of oligomeric fragments is still present. The higher the DO of the starting material was, the larger the fragments were (Fig. 1B). At a specific temperature, the level of hydrolysis can be expected to be a function of time and acid strength. In 50 mM TFA, the hydrolysis rate was relatively low; the level of hydrolysis was much lower after 8 h of hydrolysis compared to 20 h (data not shown). Non-oxidized starch was broken down completely to monomers and small oligomers under the same conditions. When the concentration of TFA used for hydrolysis is increased to 2 M (Fig. 1C), all oxidized starches are broken down to a larger extent than during the 50 mM TFA hydrolysis. Also in this case, a higher DO results in fragments with a larger hydrodynamic volume. After 8 h of hydrolysis, the sample composition was similar to the composition after 20 h (data not shown); the reaction rate is thus higher than during the 50 mM TFA incubations. Non-oxidized starch was broken down completely to monomeric glucose within 2 h. Fig. 1D reveals that oligomers are even present after a harsh acid methanolysis of oxidized starches by using 2 M HCl in methanol at 80 °C. This indicates that the linkages in $\alpha(1 \rightarrow 4)$ glucuronan are resistant to strong acidic methanolysis. The stability of aldobiouronic acids during acidic hydrolysis has been known for a long time already and can probably be ascribed to steric factors (BeMiller, 1967).

De Ruiter, Schols, et al. (1992) proposed a method in which 2 M HCl in methanol was used for degradation of uronic acid containing water-soluble polysaccharides, while a subsequent 2 M TFA hydrolysis was performed to split methylglycosidic bonds prior to HPAEC analysis. Our data imply that this method cannot be applied to $\alpha(1 \rightarrow 4)$ glucuronan. Interestingly, the fragments resulting from the incomplete degradation can be used to elucidate the structure of the oxidized starches.

3.2. Amounts of monomers released during acidic treatments

To quantify the amounts of monomers that have been released during the acidic treatments, samples were injected on HPAEC in appropriate dilutions. Fifty millimolar TFA was not sufficient to break the native starch down to monomeric glucose (Fig. 2A). Both 2 M TFA and 2 M HCl lead to a complete breakdown of native starch to monomers (data not shown for HCl). The yield in Glc after 8 h/20 h 2 M TFA hydrolysis was decreasing as a result of chemical degradation of the monomeric glucose. A release higher than ~90–95% was not reached, which means that about 5–10% of the original sample probably consisted of water or other compounds. Independent of the acid strength, it is clear that the amount of monomers released decreased when DO increased. The higher the acid strength is, the higher the amount of released monomers is, which confirms observations done after HPSEC analysis.

In Fig. 2B–D, an overview of the individual amounts of monomeric Glc and GlcA released during the several treatments of the oxidized starches can be found, expressed as a percentage of the theoretical maximum, which is 100% for each type of monomer.

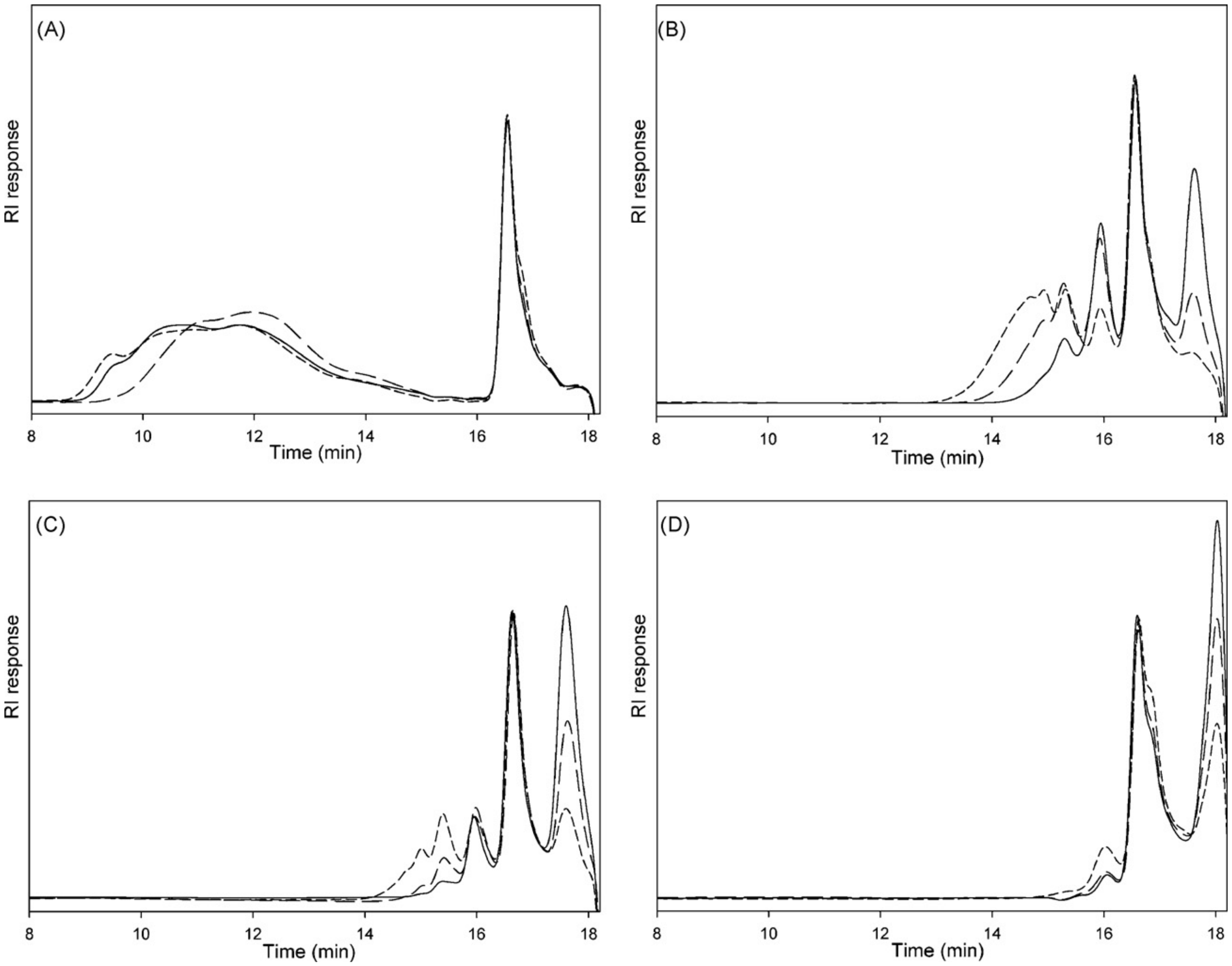

Fig. 1. HPSEC elution patterns of potato starches with DO30 (solid line), DO50 (long dash) and DO70 (short dash) prior to acidic treatment (A), after 20 h 50 mM TFA hydrolysis (B), after 20 h 2 M TFA hydrolysis (C) and after 20 h 2 M HCl methanolysis (D).

The amount of monomers released during 50 mM TFA hydrolysis decreased when DO increased and much more glucose was released compared to glucuronic acid. Only 6% of the DO 70 starch was degraded to monomers. This indicates that $\alpha(1\rightarrow4)$GlcA–GlcA bonds are hardly split and that also bonds between Glc and GlcA are hardly split by 50 mM TFA. The 2 M TFA hydrolysis and the 2 M HCl methanolysis lead to a more extensive breakdown of the oxidized starches. During the 2 M TFA hydrolysis, more glucose was released compared to glucuronic acid. This could not be quantified for 2 M HCl methanolysis, because no standard sample of the methyl glycoside of monoglucuronic acid was available. From HPSEC results it was, however, clear that the 2 M HCl methanolysis resulted in fragments with a smaller size than observed after 2 M TFA hydrolysis. Furthermore, the peak for monomeric sugars was higher after methanolysis. Especially for DO50 and DO70, the differences in monomeric glucose release are only small. It can, therefore, be concluded that in all cases relatively more monomeric GlcA O-methylglycoside is released during the 2 M HCl methanolysis than GlcA during 2 M TFA hydrolysis. This altogether means that the $\alpha(1\rightarrow4)$GlcA–GlcA bonds are resistant to acidic breakdown depending on acid nature and strength; in descending order: 50 mM TFA, 2 M TFA, 2 M HCl in MeOH. By using 2 M TFA, however, more monomeric glucose is released from the DO50 and DO70 starch compared to 2 M HCl. This means that the $\alpha(1\rightarrow4)$GlcA–Glc bond is more vulnerable to hydrolysis compared to methanolysis under the conditions used. This is probably a result of the O-methylglycosidic glucose unit at the reducing end when methanolysis is applied, which is evidently stabilizing the $\alpha(1\rightarrow4)$GlcA–Glc bond. This has to be confirmed by mass spectrometric analysis.

3.3. Oligomers released during acidic treatments

Samples were analyzed using HPAEC a second time under changed conditions to get a more detailed view on the oligomeric composition of the starch hydrolysates and methanolysates. Fig. 3 shows elution profiles of acid-treated DO30 starch (4 and 20 h of incubation).

The hydrolysis using 50 mM TFA proceeds relatively slow (Fig. 3A). Some maltodextrins are still present after 4 h of hydrolysis, and between 38 and 55 min a significant amount of large and/or densely charged fragments can be observed. It is interesting that maltodextrins up to degree of polymerization 5 are present after 4 h of hydrolysis using 50 mM TFA, although the quantity seems to be relatively low. This means that blocks of up to at least 5 non-oxidized anhydroglucose units are present within the DO30 starch polymer. After 20 h of hydrolysis, most of the late-eluting fragments have been degraded (Fig. 3B), only mono- and oligosaccharides can be observed, including some maltose. This is in line with the results of the HPSEC analysis.

The 2 M TFA hydrolysis (Fig. 3C and D) is proceeding faster; the only clear difference between 4 and 20 h of incubation is the fact that more monosaccharides have been released after 20 h. The number of peaks is lower after 2 M TFA hydrolysis compared to

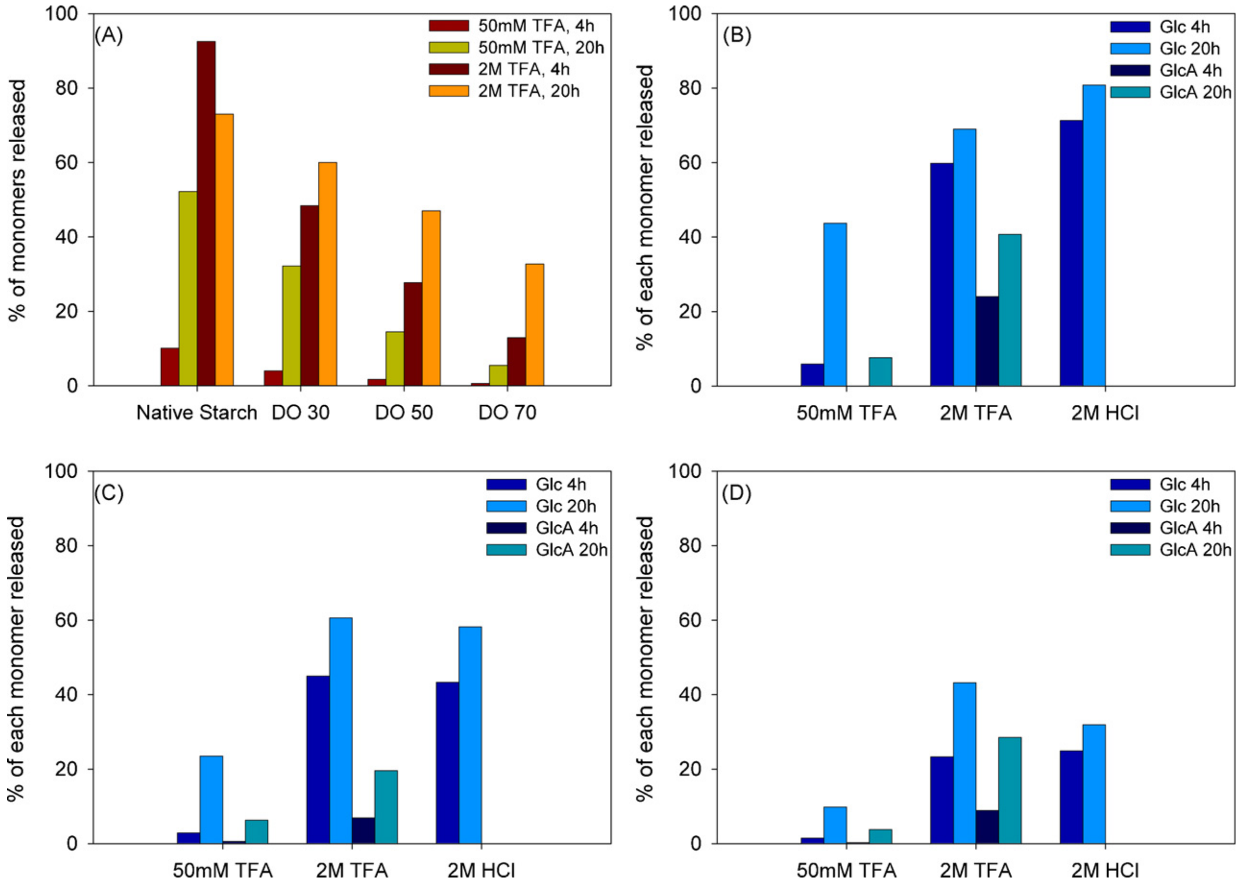

Fig. 2. Overview of the total amount of monomers (Glc and GlcA together) released from the native starch and starch with different DO during hydrolysis (A) and the individual amounts of Glc and GlcA released from the oxidized starches during hydrolysis and methanolysis (B: DO30, C: DO50, D: DO70), all expressed as the percentage of the theoretical maximum. For A, all monomers in the polymer together represent 100%; for B–D, the maximum amount of each type of monomer separately represents 100%. The amount of GlcA O-methylglycoside released during methanolysis was not quantified due to a lacking standard.

50 mM TFA hydrolysis. Because of the higher acid concentration, more bonds can be hydrolyzed, which apparently leads to the formation of a less disperse set of products.

The apparent sample composition after methanolysis using 2 M HCl (Fig. 3E and F) is completely different from the sample composition after hydrolysis. The methyl–glycosidic bonds present in each fragment lead to a shift of the peaks towards shorter elution times, which makes the chromatograms somewhat difficult to compare. However, it can be observed that 2 M HCl methanolysis leads to products with a lower size compared to TFA hydrolysis, as observed before using HPSEC. The similarity between the samples after 4 and 20 h of incubation shows that after a 4-h 2 M HCl methanolysis the endpoint of the reaction has already been reached.

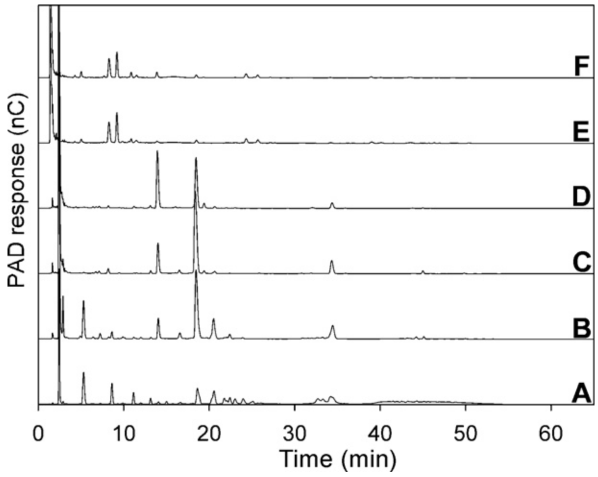

Fig. 3. HPAEC elution profiles of DO30 starch after 50 mM TFA hydrolysis (4 h: A, 20 h: B), 2 M TFA hydrolysis (4 h: C, 20 h: D) and 2 M HCl methanolysis (4 h: E, 20 h: F). Methyl α-D-glucopyranoside is eluted after 1.5 min, glucose after 2.5 min, and glucuronic acid after 13.5 min.

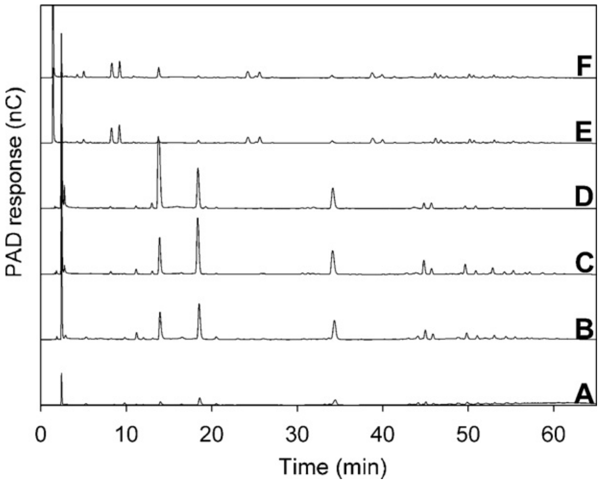

Fig. 4. HPAEC elution profiles of DO70 starch after 50 mM TFA hydrolysis (4 h: A, 20 h: B), 2 M TFA hydrolysis (4 h: C, 20 h: D) and 2 M HCl methanolysis (4 h: E, 20 h: F). Methyl α-D-glucopyranoside is eluted after 1.5 min, glucose after 2.5 min, and glucuronic acid after 13.5 min.

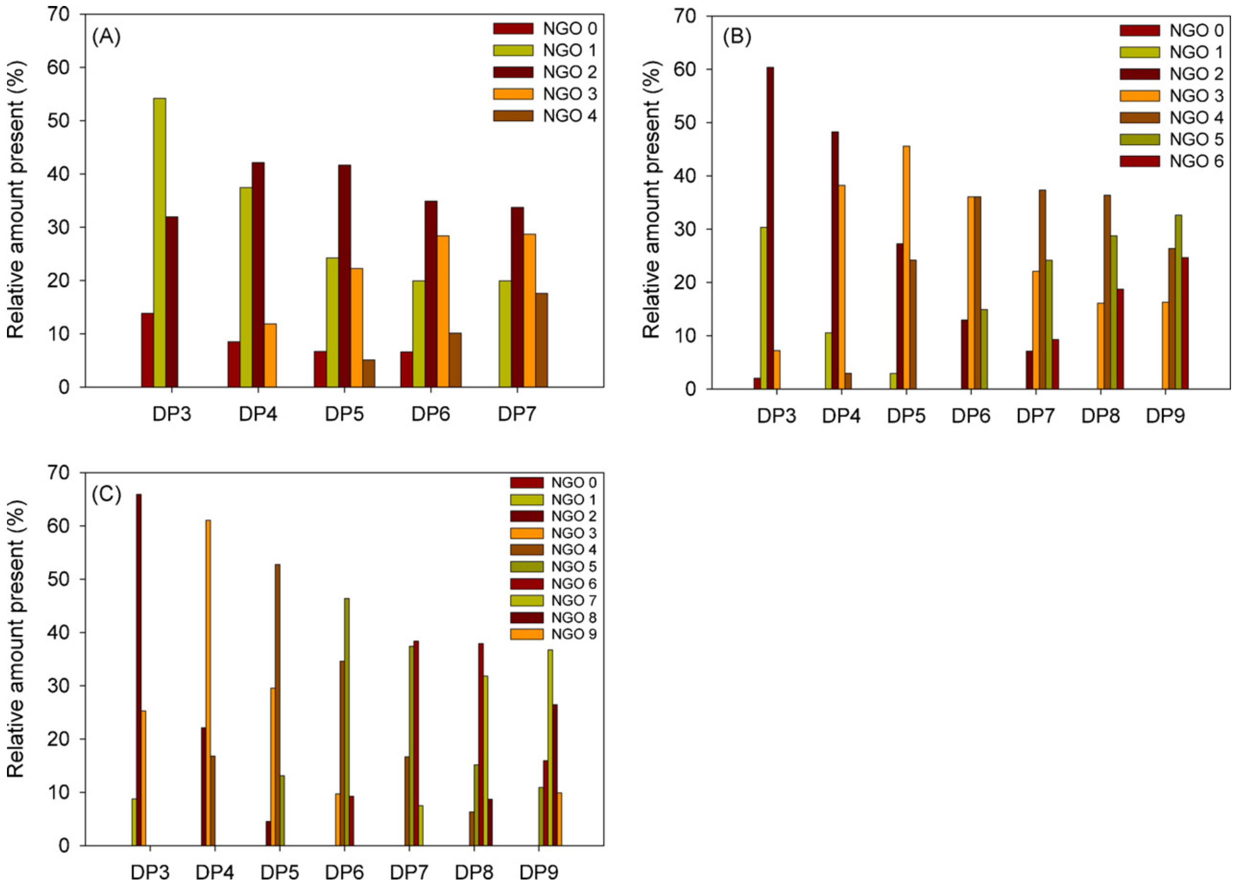

Fig. 5. Sample composition between *m*/*z* 500–3000 after 20 h hydrolysis using 50 mM TFA of DO 30 starch (A), DO 50 starch (B), and DO 70 starch (C), based on MALDI-TOF MS analysis. NGO = number of GlcA moieties per oligomer. The total amount of each DP together represents 100%.

Fig. 4 shows the HPAEC elution profiles of the hydrolyzed/methanolyzed DO70 samples. The most striking differences between Figs. 3 and 4 are within the >35 min elution time region. In the DO70 samples, many late-eluting oligomers are present after hydrolysis/methanolysis, which indicates that they are either large or heavily charged, or both. Apparently, the distribution of the GlcA moieties makes a complete breakdown to small oligomers or monomers impossible. For all DOs, even a one-hour 2 M TFA hydrolysis step after 20 h of 2 M HCl hydrolysis, as proposed by De Ruiter, Schols, et al. (1992), does not affect the backbone of these oligomers (data not shown). This again underlines the conclusion that α(1 → 4) linked D-glucuronan segments are very resistant to acidic degradation, even under severe conditions. Compared to results obtained in pectin research, these results are remarkable, as this type of acidic degradation is favorably used for determination of the monomeric composition of uronic acid containing polymers. Very recently (Willför et al., 2009), a combination of methanolysis and hydrolysis was again recommended for analysis of the sugar composition of, e.g. pectins. However, this treatment appears to be not sufficient for breakdown of oxidized starches.

Polymeric glucuronans have previously been isolated from moulds after a treatment with 2 M HCl at 100 °C for 4 h (De Ruiter, Josso, Colquhoun, Voragen, & Rombouts, 1992). This is in line with our observations, although these glucuronans consisted of β(1 → 4) linked uronic acid moieties. The resistance to harsh acidic conditions apparently also accounts for α(1 → 4) linked uronic acid moieties present in TEMPO-oxidized starch, but not for α(1 → 4) linked galacturonic acid moieties.

The presence of large oligomers after the acidic treatments furthermore indicates that the distribution of GlcA moieties in starch with a high DO is blockwise. A randomly oxidized starch molecule would at least be degradable to smaller oligomers than observed in these HPAEC spectra. Considering the complete breakdown of native starch, it can be assumed that all Glc-(1 → 4)-Glc bonds are degraded during the 2 M HCl and 2 M TFA treatments. The Glc-(1 → 4)-GlcA bond can also be expected to be degraded, while the GlcA-(1 → 4)-Glc bond can be expected to be more resistant to the acidic treatment. The same was observed when a polymer containing galacturonic acid (GalA) and galactose (Gal) moieties was degraded by using HF, resulting in GalA-(1 → 4)-Gal aldobiouronic acids and monomeric Gal (Mort, Qiu, & Maness, 1993). This implies that – in our case – glucosyl linkages will be more vulnerable to lysis than glucuronosyl linkages. The more random the distribution of GlcA moieties is, the more uniform the set of degradation products would be. In our case, however, larger oligomers of varying size, which probably mainly consist of GlcA moieties, seem to be present in the polymer. Mass spectrometric techniques will provide more insight into the exact structure of the smaller oligomers (<3000 Da): the results of this analysis will be discussed later. The larger oligomers (>3000 Da) will not be observed by using MS. The fact that these large oligomers are still present after, e.g. the 2 M TFA hydrolysis process indicates that these fragments should consist of clustered GlcA moieties, considering the findings with regard to the vulnerabilities of the several bonds present in the polymer, as mentioned above.

3.4. Fragment structure determination using MALDI-TOF MS

MALDI-TOF MS analysis was performed to obtain information about the structure of the individual oligomers, and thus about the hydrolysis/methanolysis process, and the structure of the oxidized

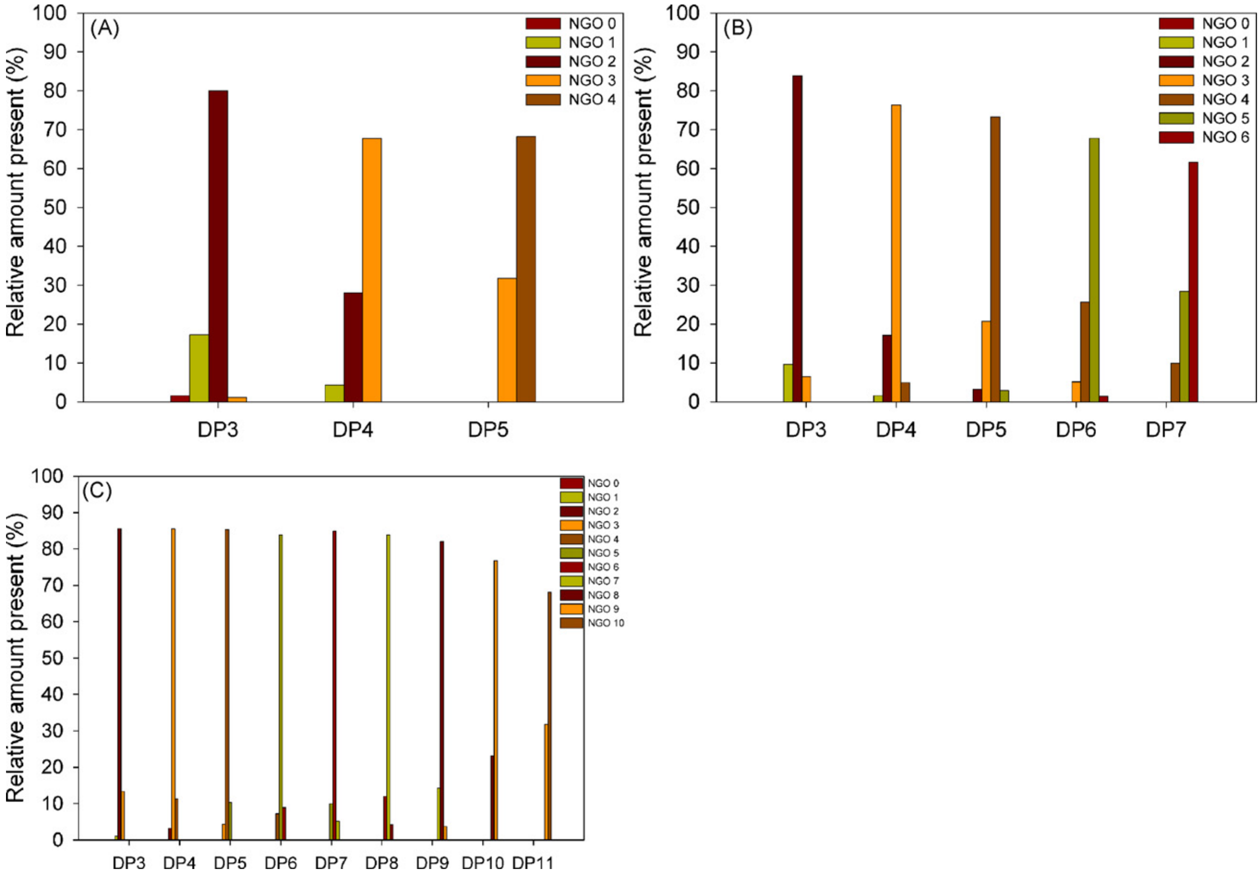

Fig. 6. Sample composition between m/z 500–3000 after 4 h methanolysis using 2 M HCl of DO 30 starch (A), DO 50 starch (B), and DO 70 starch (C), based on MALDI-TOF MS analysis. NGO = number of GlcA moieties per oligomer. The total amount of each DP together represents 100%.

starch polymers. Results will be discussed on the basis of the acidic treatment.

3.4.1. *Structure of oligomers present after 50 mM TFA hydrolysis*

In Fig. 5, the oligomeric composition of the samples after 20 h 50 mM TFA hydrolysis can be found. The hydrolysate of DO30 starch contains maltodextrins with DPs up to 6 (Fig. 5A). After 4 h of hydrolysis, even maltodextrins with DP 7 were observed. This indicates the presence of blocks of non-oxidized anhydroglucose units in the polymer. Furthermore, on average not more than half of the sugar moieties present in the oligomers are GlcA moieties. This relative number of GlcA units present in the oligomers decreases when DP increases, while the variety in oligomers present increases. This indicates that the distribution of oxidized groups within the individual oligomers is quite random, assuming that Glc–Glc bonds are, in general, the most vulnerable to acidic hydrolysis. If the GlcA units were present in blocks, smaller oligomers would have been formed. This would yield a higher average number of GlcA per oligomer.

When polymers with DOs 50 and 70 are hydrolyzed using 50 mM TFA (Fig. 5B and C), it appears that the size of the fragments does not increase very much compared to the DO30 samples. The most important trend is that the average number of oxidized groups per oligomer increases with increasing DO. Even fragments solely consisting of GlcA units can be observed in the DO 70 hydrolysate. Furthermore, the relative amount of released monomeric GlcA decreases when DO increases (Fig. 2). This means that the distribution of GlcA units along the polymer has an increasing level of blockiness when DO increases. TEMPO apparently prefers to act in regions were oxidized groups are already present. The fact that the part of the polymer that is degraded to small oligomers decreases when DO increases, as observed in HPSEC analysis, supports this statement.

A restriction of the MALDI-TOF MS analysis is the limited m/z range, which causes the analysis of only a part of the molecules present in each sample. The part of the polymer observed as oligomer in MS analysis can, however, be estimated based on the monomer quantification and on HPSEC results. Depending on the DO, 5–30% of the starch was recovered as monomer (Fig. 2A). Based on HPSEC calibration, it can, furthermore, be calculated that compounds eluting >15 min are observed in the MALDI-TOF MS analysis (data not shown). When these data are combined with HPSEC (Fig. 1B), the oligomers in Fig. 5 can be estimated to represent the following part of the original oxidized starches: DO30: 55%, DO50: 55%, and DO70: 40%. It is hard to draw strong conclusions on the structure of the 15–50% of the polymer that was not observed, since not even all Glc–Glc bonds present in native starch were hydrolyzed in 50 mM TFA. The reduced degradability when DO increases, however, suggests that at least a significant number of clustered GlcA units are present in these relatively large oligomers.

3.4.2. *Structure of the oligomers present after 2 M TFA hydrolysis*

The 2 M TFA treatment mainly yields oligomers consisting of GlcA units carrying one Glc unit, the size of these oligomers increases when DO increases. After 20 h, oligomers solely consisting of GlcA units are present, they represent about 30% of the DP 3–7 oligomers present in the DO70 hydrolysate (graphs not shown). From Fig. 2A it is known that 30–60% of the polymer is degraded to monomers (depending on DO). It can then be roughly estimated based on HPSEC results (Fig. 1C) that the oligomers that were actually observed in the MALDI-TOF MS analysis approximately represent the following percentage of the original oxidized

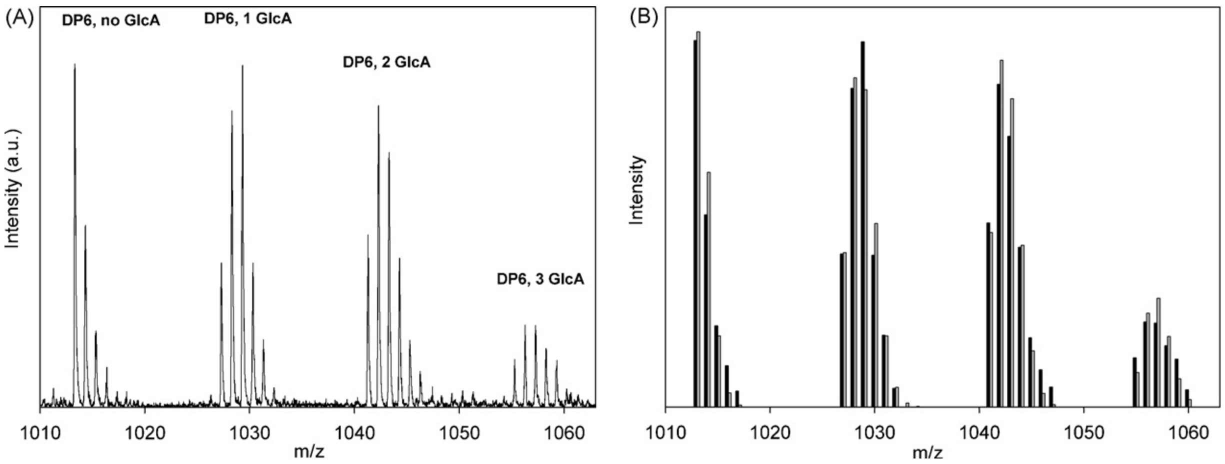

Fig. 7. Zoom-in on the DP6-region of the MALDI-TOF mass spectrum of the 50 mM TFA hydrolysate of DO 30 starch after 4 h of incubation (A) and this measured isotopic distributions (black) combined with the calculated isotopic distributions showing the best fit (gray) in a bar-graph (B). The original maltohexaose has an *m*/*z* value of 1013. An increase of this *m*/*z* value to 1027, 1041, and 1055 can be observed, which indicates the presence of 1, 2, and 3 GlcA moieties in the oligomer, respectively.

polymers: DO30: 35%, DO50: 40%, and DO70: 40%. This means that these results provide a realistic view on the structure of the original polymer. The part of the oligomer that is not observed consists of structures that are highly resistant to 2 M TFA hydrolysis, which are most probably large blocks of GlcA moieties. These results confirm the increasing blockiness with increasing DO.

3.4.3. Structure of oligomers present after 2 M HCl methanolysis

In Fig. 6, the composition of the samples after 4 h 2 M HCl methanolysis can be found. The time point of 4 h was chosen to minimize the effect of GlcA–GlcA bonds that had been split. Based on HPSEC results, it is known that all fragments are within the *m*/*z* range of this MALDI-TOF MS analysis, even after 4 h of incubation (data not shown). Therefore, these fragments, in combination with the monomer analysis, provide a realistic view on the structure of the original polymer. The maximum number of oxidized groups observed per oligomer is about the same as after 50 mM TFA hydrolysis, but the proportion of oxidized moieties per oligomer is much higher. Furthermore, the maximum DP of the oligomers increases when DO increases. The fact that the maximum size of GlcA blocks that can be observed is increasing when DO increases (DO 30: 4, DO 50: 6, and DO 70: 10) indicates that the degree of blockiness is increasing when DO increases. In the very first stage of the oxidation process, TEMPO is probably randomly acting at different places in the polymer. When the oxidation proceeds, TEMPO apparently has a preference to act close to groups that have already been oxidized. This causes the presence of relatively small GlcA blocks in the DO 30 starch, the size of these blocks is then increasing when DO increases, as observed in Fig. 6.

MALDI-TOF MS analysis furthermore suggests that the $\alpha(1 \rightarrow 4)$ GlcA–Glc bond is more vulnerable to hydrolysis compared to methanolysis. During 2 M HCl methanolysis of DO70 starch, hardly any oligomers solely consisting of GlcA were formed. Only some low DP GlcA oligomers were observed after 20 h of incubation (data not shown), probably as a result of GlcA–GlcA bonds being split. Two moles TFA hydrolysis for 20 h did release relatively more oligomers solely consisting of GlcA from DO70 starch, as discussed before. The O-methylglycosidic glucose moiety apparently increases the resistance of the $\alpha(1 \rightarrow 4)$ linkage with the GlcA moiety to acidic breakdown.

3.5. Abundance and location of aldehydo groups after starch oxidation

During the oxidation reaction, hydroxyl groups are converted into aldehydes by activated TEMPO. These aldehydes are subsequently converted to carboxyl groups by OCl^-. As determined in a kinetic study using methyl-α-D-glucopyranoside as a substrate (De Nooy, Besemer, & van Bekkum, 1995b), the latter is the most rapid reaction. The maximum concentration of aldehydes that can be expected in the reaction medium is, however, still about 10%. The amount of OCl^- used in our experiments indicates that these data are also applicable when starch is oxidized (data not shown). After oxidation, the remaining aldehydo groups, which make the polymer unstable, were reduced to hydroxyl groups. For this, $NaBD_4$ was used instead of the usual $NaBH_4$, $NaBD_4$ causes the incorporation of a deuterium atom instead of a hydrogen atom at the location of the reduced aldehydo groups. These locations are then visible in subsequent mass spectrometric measurements. In Fig. 7A, a zoom-in of a MALDI-TOF mass spectrum after 4 h of 50 mM TFA hydrolysis of DO 30 starch can be found. This sample was chosen because it also contains oligomers which do not contain GlcA moieties. The zoom-in shows the maltohexaose without any oxidized units. Furthermore, oligomers with DP 6 containing 1, 2, and 3 GlcA moieties can be observed in the spectrum. The isotopic distribution of the oligomers without any GlcA moieties seems to be normal, while this distribution changes when one or more oxidized groups are included. A similar trend was observed for other DPs. To determine

Table 1
Overview of the contribution of each type of DP6 oligomer to the total amount of DP 6 oligomers in the DO30 sample after 4 h of hydrolysis using 50 mM TFA. Furthermore, the average amount of deuterium atoms relative to (i) the number of Glc and (ii) the total 6 moieties, in each type of oligomer, are provided. In the final column, the average percentage of moieties in the oligomer converted to either GlcA or and aldehyde are provided for each type of DP6 oligomer. Numbers are based on the data in Fig. 7.

DP6 with NGO	% of total DP6 oligomers (amount)	% of remaining Glc carrying a deuterium atom	% of 6 moieties carrying a deuterium atom	% of 6 moieties converted to GlcA/aldehyde
0	22	3	3	3
1	34	20	16	33
2	33	21	14	47
3	11	32	16	66

how many deuterium atoms are on average present in each of the DP 6 oligomers, calculations were made in which the abundance of the natural ^{13}C isotope was taken into account. The percentage of anhydroglucose units carrying a deuterium atom was varied, and the best fit with the measured values was determined. In Fig. 7B, this measured isotopic distributions are combined with calculated isotopic distributions which showed the best fit, in a bar-graph. In Table 1, the contribution of each type of DP6 oligomer to the total can be found. Furthermore, the average amount of deuterium present in these oligomers can be found, expressed relative to number of Glc units present and relative to the total 6 moieties present in the oligomer. In the last column, the total percentage of moieties converted to either GlcA or an aldehyde can be found.

The oligomers that are not carrying any GlcA unit show a minor amount of deuterium groups (3%), which means that hardly any aldehydo groups have been present in the region where these oligomers result from. The amount of deuterium present in the oligomers, relative to the number of Glc moieties present, substantially increases when one GlcA unit is present, and it further increases when more GlcA units are present. This indicates that a relatively large amount of aldehydes has been present in the regions where GlcA units are present. On average, one aldehydo group (∼16%) has been present in each DP6 oligomer containing one or more GlcA units. This illustrates that TEMPO prefers to act in a region where GlcA moieties are present already, regardless of the exact number of GlcA present.

These observations can only be explained by a strongly clustered TEMPO oxidation and subsequent conversion to GlcA already in the initial phase of the starch oxidation process. Once TEMPO is active in a certain region of the polymer, it tends to keep on being reactive in this region. The explanation of this probably lies in the fact that the oxidized form of TEMPO, which is the oxidizing agent, is the positively charged nitrosonium ion. Conversely, the carboxyl groups are negatively charged at the pH applied. The attraction between these charges causes the action of the nitrosonium ion close to regions that contain one or more GlcA moieties. This leads to clusters of GlcA units and Glc units carrying an aldehydo group on the one hand, and clusters of non-modified Glc units on the other hand.

4. Conclusions

The $\alpha(1\rightarrow4)$ GlcA–GlcA bond present in TEMPO-oxidized potato starch was found to be very resistant to acidic degradation, while the $\alpha(1\rightarrow4)$ GlcA–Glc bond also showed increased resistance compared to the $\alpha(1\rightarrow4)$ Glc–Glc bonds. This will on the one hand lead to incorrect results when these methods are used for analysis of the monomeric composition of oxidized starches. On the other hand, the oligomers obtained after acidic fragmentation could be applied to elucidate the structure of these modified biopolymers. It was found that TEMPO oxidation of starch leads to the formation of blocks of GlcA within the polymers. The size of these blocks increases when the degree of oxidation of the polymer increases. Apparently, the TEMPO radical tends to be active close to groups that have already been converted to GlcA. This can be explained by the negative charge carried by the carboxyl groups at the pH applied during the oxidation process; this attracts the positively charged activated TEMPO. This non-random action of TEMPO was confirmed by the presence of aldehydes clustered around regions were GlcA units were detected, just after the oxidation process.

To date, the distribution of the GlcA units along the backbone of TEMPO-oxidized starch was unknown, while this distribution can be expected to have an impact on the functionality of the polymer. This is also the case, for example, in citrus pectins. The distribution of methylated, uncharged groups along the charged polymer, indicated by the degree of blockiness, had a significant impact on its gelling behavior at different pH and Ca^{2+} levels (Lofgren, Guillotin, Evenbratt, Schols, & Hermansson, 2005). Our findings may, therefore, lead to an improved functionality of TEMPO-oxidized starch polymers.

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