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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 $\alpha(1 \rightarrow 4)$ glucuronic acid–glucuronic acid bond was found to be very resistant to breakdown by acids. The $\alpha(1 \rightarrow 4)$ glucuronic acid–glucose bond also showed increased resistance to acids compared to $\alpha(1 \rightarrow 4)$ 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 molec-