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Preparation and analytical characterisation of pure fractions of cellooligosaccharides

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ABSTRACT

In the present work, a viable protocol was developed to prepare monodisperse cellooligomers up to a degree of polymerisation (DP) of 20. Peracetylated cellooligosaccharides were obtained from cellulose by acetolysis and subsequently purified by Normal Phase-High Performance Liquid Chromatography using toluene, ethyl acetate and acetone as eluents. In addition, we demonstrated how to efficiently monitor the purity and dispersity of the obtained compounds by High Performance Thin Layer Chromatography, Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry and Nuclear Magnetic Resonance. With this approach, it is possible to isolate cellooligomer standards up to DP 20 on a preparative scale (dozens of mg). The column chromatographic separation proved to be robust over several months and to be scalable from a analytical to a preparative column. The isolated oligomer standards allow a more precise description of cellooligomer distributions typically emerging from biorefinery process streams after hydrolysis of lignocelluloses. They can be used to calibrate the oligomeric region in size-exclusion chromatography where light scattering detection fails due to limited scattering intensities.

1. Introduction

Cellooligosaccharides – also known as cellodextrins – are linear chains of β-1→4 linked anhydroglucose units with a low degree of polymerisation (DP). They can be considered short cellulose molecules [1]. A clear demarcation between cellulose polymers and cellooligosaccharides (COS) does not exist; commonly, a DP of 5–7 is proposed as upper limit, since these are the largest COS that are still water soluble [2,3]. In contrast to cellulose, the chemistry of COS is still in its infancy, mostly due to the non-availability of sample material. It is foreseeable that higher DP COS samples would serve well as cellulose models in studies on cellulose crystallinity, cellulase activity, hydrolytic cleavage and the change in physicochemical properties with DP [4–6]. Current industrial applications can be found in the food and pharmaceutical industries [7], yet future biorefineries based on the hydrolysis of lignocelluloses to glucose will certainly encounter COS originating from an incomplete hydrolysis of their feedstock.

Appropriate analytical techniques need to be available to determine COS, for example, during process control in a biorefinery process. Instrumental analytical methods capable of separating and detecting COS exist, namely size-exclusion chromatography, normal phase-high performance liquid chromatography (NP–HPLC), hydrophilic interaction liquid chromatography, ion exchange chromatography and affinity chromatography. However, standards to calibrate these methods are to a large extent unavailable; commercial offers, which often exhibit limited purity, end at DP 7 [8]. In general, COS can be obtained either by synthesis starting from the monomer or dimer or by degradation of the polymer. Synthetic polymerisation of polysaccharides is extremely difficult, since a specific new bond has to be formed both regioselectively and stereoselectively [9]. For enzymatic polymerisation, the obstacles include purification of the enzymes, preparation of a favorable substrate and the insolubility of COS with a DP above 8 in water [10]. Enzymatic degradation offers similar challenges. Chemical degradation of cellulose can be achieved with different acids, often at low temperatures to prevent by-product formation [11]. The degradation of cellulose with acetic acid in the presence of acetic anhydride and sulfuric acid yields peracetylated cellooligomers (PCOS) [2,12–14]. Depending on the reaction time, different distributions of DPs can be obtained. The reduced hydrophilicity of the

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PCOS due to peracetylation renders them soluble in organic solvent and therefore allows their purification by normal phase chromatography. For a comparison of several pathways for generating xylo- and cellooligomers, the reader is referred to a recent review [15].

In this work, an efficient technique for preparing pure, monodisperse cellooligomers up to DP 20 in the scale of several tens of milligrams is described. To our knowledge, previous approaches to isolate COS yielded approximately 10 mg of COS with a DP of up to 5 [11]. However, Arndt et al. [16] were able to prepare PCOS up to a DP of 9 in gramme scale, using pivalolysis and cellulose tri-acetate as starting material. Conditions of acetolysis have been adapted to obtain a PCOS fraction between DP 10 and 20. Methods were developed for qualitative high-performance thin-layer chromatography (HPTLC), and analytical and preparative-scale NP–HPLC for reaction control and purification of the obtained PCOS. The identity and purity of the PCOS were controlled by HPTLC-matrix-assisted laser desorption/ionization-time of flight (MALDI–TOF)-mass spectrometry (MS), nuclear magnetic resonance (NMR) and offline MALDI–TOF–MS. COS can be released from PCOS by simple deacetylation. The availability of COS standards in these amounts and up to DP 20 allows for the development of further analytical methods.

2. Materials and methods

2.1. Chemicals

Acetonitrile (liquid chromatography [LC]-MS Chromasolv grade), acetone (>99.8%), acetic anhydride, trifluoroacetic acid (TFA, Chromasolv grade), sodium methanolate (0.5 M in methanol), sodium bicarbonate, ammonium dihydrogen phosphate (>98.5%), microcrystalline cellulose (Avicel PH101, 50 μm), *p*-anisaldehyde (98%) and cellobiose octaacetate were purchased from Sigma–Aldrich/Fluka (Schnelldorf, Germany). Ethyl acetate, toluene, sulphuric acid, 2,5-dihydroxybenzoic acid (DHB, >99%), silica 60 (40–63 μm) and TLC-plates – for HPTLC: HPTLC silica 60 F_{254}, 200 μm thickness, 10 × 10 cm, glass backed (No. 1.05628.0001); for MALDI: silica 60 F_{254}, 200 μm thickness, 5 × 7.5 cm, aluminium backed (No. 1.05549.0001) – were obtained from Merck (Darmstadt, Germany). Acetone, glacial acetic acid and hydrochloric acid (HCl, 37%) were provided by VWR (Vienna, Austria). α-D-Glucose pentaacetate was obtained from Alpha Aesar (Karlsruhe, Germany). Sulphuric acid (96%, p.a.) was purchased from Roth Lactan (Graz, Austria). Black ribbon filter paper, MN640w, No. 41, was obtained from Macherey & Nagel (Düren, Germany).

2.2. Reactions

2.2.1. Acetolysis

Acetolysis was conducted following Hess and Dziengel [12] with slight adaptations [17]: In this protocol, 45 g of cellulose was added to 170 mL of glacial acetic acid, 170 mL of acetic anhydride and 36 mL sulphuric acid in a beaker equipped with a mechanical stirrer set at 750 rpm. The reaction was cooled with an ice bath during dissolution and then stirred at room temperature. After the chosen reaction time, the clear yellow solution was filtered through a sintered glass funnel. The filtrate was slowly poured into 2 L of ice-cold water under mechanical stirring to precipitate the generated PCOS. The resulting suspension was carefully neutralised with sodium bicarbonate and then stored at room temperature overnight. The precipitate was isolated via suction filtration through black ribbon filter paper. The solid product on the filter was washed with deionised water until the filtrate had a neutral pH. Then, the solid was dried to constant weight in a vacuum desiccator over anhydrous calcium chloride, powdered in a mortar and stored in a desiccator over solid sodium hydroxide and anhydrous calcium chloride. With this method, 85% of the cellulose could be converted into PCOS and recovered, assuming a typical increase of molar mass by 80% by acetylation.

2.2.2. Zemplén deacetylation

Cellooligomers were obtained by deacetylation following Zemplén [18]. Ten milligrammes of the acetylated cellooligomers were mixed with 4 mL of sodium methanolate (0.5 M in methanol) and stirred for 2 h at room temperature. The reaction was then neutralised with HCl (0.5 M).

Solutions of cellooligomers with a DP up to 6 were lyophilised directly after deacetylation. Thus, a considerable amount of sodium chloride remained in these samples, which would have to be removed for applications sensitive to inorganics.

Suspensions of cellooligomers with a DP above 6 were centrifuged (15 min at 1420 × *g*) to obtain a pellet of insoluble oligomers, which was washed three times with hot water at 60 °C and then lyophilised.

2.3. Chromatography

2.3.1. High-performance thin-layer chromatography (HPTLC)

Samples were spotted on the TLC plate with an Automatic TLC Sampler (ATS 4, CAMAG, Muttenz, Switzerland). TLC plates were developed with ethyl acetate:toluene 8:2 (v/v) in a CAMAG twin-trough 10 × 10 cm chamber without chamber saturation to a migration distance of 60 mm.

For visualisation, the plate was immersed manually into anisaldehyde reagent (1 mL of *p*-anisaldehyde in 170 mL methanol with 20 mL glacial acetic acid and 10 mL sulphuric acid (96%); stable up to 6 weeks at 4 °C). After heating the plate on a glass-ceramic hot plate (11 A, Harry Gestigkeit GmbH, Karlsruhe, Germany) at 120 °C for 5 min, images were taken with a TLC Visualizer (CAMAG, Muttenz, Switzerland) with the following settings: white light, transmission mode, auto exposure, gain 1. CAMAG instruments were controlled with VisionCats v1.4. (CAMAG, Muttenz, Switzerland).

2.3.2. Analytical high-performance liquid chromatography (HPLC)

The HPLC system consisted of a Merck Hitachi L-6200 ternary pump equipped with a 30 mL min^{-1} pump head; a manual 6-port Rheodyne injection valve (VICI AG International, Schenkon, Switzerland) equipped with a 20, 30 or 100 μL injection loop; a Kromasil 100, 7 μm, 250 × 4.6 mm column (MZ-Analysentechnik, Mainz, Germany); and an evaporative light scattering (ELS) detector (Sedere Sedex 90LT, Olivet, France) with the following parameters: 3.5 bar nitrogen, nebuliser flow range 250 μL-2.5 mL min^{-1}, 58 °C, gain: 8.

The flow rate was set to 0.6 mL min^{-1} with a gradient from 50% B to 100% B in 90 min, then 10 min 50% B. Solvent A was toluene; solvent B was ethyl acetate with 1% (v/v) acetone.

To prevent column blockage, the samples had to be prepared for analysis as follows: One gramme of solid PCOS was dissolved in a suspension of 1 g of silica in 20 mL of acetone. After stirring for 30 min at room temperature, acetone was carefully evaporated *in vacuo*. The PCOS adsorbed on the silica were redissolved by stirring at room temperature for 30 min in 10 mL ethyl acetate with 1% acetone/toluene 1:1 (v/v). Then, the obtained solution was filtrated first through a sintered glass funnel and then through a 0.45 μm disposable PTFE-syringe filter. The obtained PCOS concentration was found to be 7.8 mg mL^{-1} by evaporation.

2.3.3. Preparative HPLC

The preparative HPLC system comprised a Knauer 1800 binary low-pressure gradient pump with a 250 mL min^{-1} pump head and