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Short communication

Analysis of isoamylase debranched starches with size exclusion chromatography utilizing PFG columns

Jelena Ciric[a,b], Albert J.J. Woortman[a], Katja Loos[a,b,*]

[a] Department of Polymer Chemistry & Zernike Institute for Advanced Materials, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands
[b] Dutch Polymer Institute (DPI), P.O. Box 902, 5600 AX Eindhoven, The Netherlands

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ABSTRACT

Debranched starches were tested with a previously developed method for size exclusion chromatography (SEC) with multi detection utilizing different columns than usually used for the separation of starch in DMSO. A number of debranched starches were analyzed. This system allows good separation of amylose and amylopectin after debranching of starch, and provides quantitative information on the amylose content. Additionally molar mass versus hydrodynamic radii (R_h) distributions of various debranched starches show that the debranching was not 100% and that the differences in the structure of various starches can be followed.

1. Introduction

Starch is the most consumed polysaccharide in human diet. It consists of two main glucose polymers, mostly linear amylose (molecular weight $\approx 10^6$) and branched amylopectin (molecular weight $\approx 10^8$). Therefore, it is very complex concerning its molecular architecture, whereas very simple concerning its chemical structure (the monomer is glucose) (Buléon, Colonna, Planchot, & Ball, 1998; Ciric, Petrovic, & Loos, 2014). The molecular architecture (the molar mass/size distributions of starch molecules, their components and branches) and the ratio of the two main components of starch are very important due to extensive use of starch in non-food industry and the impact they have on the digestibility and nutritional values, cooking and processing quality, or end-use of the products made of starch (Ahmadi-Abhari, Woortman, Hamer, & Loos, 2013). Determination of the different distributions is one of the most perplexing tasks in starch characterization. Difficulties occur due to broad distributions and structural complexity (Roger, Tran, Lesec, & Colonna, 1996; Yu & Rollings, 1987). The most developed technique for determination of the size distributions of starch and starch-like polymers is size-exclusion chromatography (SEC)(Hoang et al., 2008; Yokoyama, Renner-Nantz, & Shoemaker, 1998; Vilaplana, Hasjim, & Gilbert, 2012; Vilaplana & Gilbert, 2010; Gaborieau & Castignolles, 2011).

Much research has been performed on this topic, nevertheless a full description of these complicated structures is lacking for many reasons, which include solubility problems, degradation during size separation, the broadness of size distributions, column interactions, etc. One of the most complete characterizations of natural starches was achieved when the first two-dimensional structural distribution for starch was established in 2010, in which the weight distribution of whole starch molecules as a function of hydrodynamic size and of the hydrodynamic size of individual branches was presented (Vilaplana et al., 2012). This technique combined size fractionation by preparative SEC, collection of size-separated fractions, enzymatic debranching of these fractions with isoamylase, and analysis of the branched and debranched fractions in DMSO by analytical SEC with multi detection and GRAM (polyester copolymer network PSS, Mainz, Germany) columns. It was shown that the results of the 1D branch chain length distribution and those from the 2D distribution (where unambiguous separation between amylopectin and amylose existed) resulted in acceptable agreement for the amylose content, with a moderate underestimation in the 1D measurements.

In our previous work, we showed that the use of PFG (modified silica; PSS, Mainz, Germany) columns has the potential for a very good separation and analysis of linear and branched synthetic starch-like polysaccharides, when DMSO/LiBr is used as a solvent (Ciric, Oostland, Vries, Woortman and Loos, 2012). This work helped in understanding synthesis-molecular

* Corresponding author. Tel.: +31 50 3634400.
E-mail addresses: jelenaciricnl@gmail.com (J. Ciric), a.j.j.woortman@rug.nl (A.J.J. Woortman), k.u.loos@rug.nl, katjaloos@web.de (K. Loos).

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architecture–property relations of starches. Additionally, due to the previously shown good separation this system could be easily used for determination of the amylose content of starch.

Therefore we wanted to expand this study, and test our system with natural starches. However, due to band broadening, shear scission and a large overlap between the small amylopectin molecules and the amylose, 1D whole-molecule size distribution significantly overestimates the amylose content (Vilaplana et al., 2012). To overcome this problem the size/molecular weight distribution of the branches can be obtained by enzymatic debranching of starch: each branch point is enzymatically cleaved by a standard technique. The resulting material is composed of mostly linear polymer chains, which we denote as linear starch, and avoids band broadening and shear scission due to reduced size (Gidley et al., 2010). Hence, we decided to test our system with this 1D branch chain length distribution method for the amylose content determination. Since the molecular architecture of starches is very important for their final use, we additionally checked the size distribution of debranched starches and the dependence of molar mass on hydrodynamic radius (R_h) (Bhattacharya & Corke, 1996). These distributions affect viscosity and texture of the final products (Tokimura et al., 2002). We also observed that the slight difference in molar mass for the same size of different starches does exist, even after debranching. This indicates an incomplete debranching with the used enzyme. Nevertheless, in this way it is possible to follow the difference in the structure of starches.

2. Materials and methods

Glucose-1-phosphate (G-1-P), (tris(hydroxymethyl)aminomethane) (Tris), dithiothreitol (DTT), adenosine monophosphate (AMP), phosphorylase *b*, isoamylase from *Pseudomonas sp*, DMSO, NaN_3, potato starch, wheat starch, corn and Waxy corn starch were purchased from Sigma–Aldrich. LiBr was purchased from Fisher Scientific, Hylon VII from National Starch & Chemical Co, Eliane from Avebe food, whereas maltoheptaose (G-7), and glycogen branching enzyme from *Deinococcus geothermalis* (Dg GBE) were synthesized or expressed and isolated as stated elsewhere.

2.1. Synthesis of well-defined linear and branched polysaccharide (Ciric, Oostland, de Vries, Woortman, & Loos, 2012; Ciric & Loos, 2013)

G-1-P was dissolved in Tris buffer (100 mM, pH 6.7, 0.02% NaN3) containing G-7 (0.7 mM) as a primer, DTT (1.3 mM) as a reducing agent, and AMP (3.5 mM) as a phosphorylase *b* activator, and the pH was adjusted to 7. The polymerization was catalyzed by addition of rabbit-muscle phosphorylase *b* (1.5 U/mL) and the branching was initiated by Dg GBE (250 U/mL) at 37 °C. The reaction time was 6 h, and the concentration of G-1-P was 350 mM. Termination was done by a 5 min heat-treatment. The samples were dialyzed (3500 MWCO) to remove the excess of G-1-P, AMP, and DTT, and freeze-dried.

2.2. Debranching of the polysaccharides (van der Vlist et al., 2008)

With final concentration of 10 mg mL^{-1} each starch was suspended in citrate buffer (1 M, pH 4.0, 0.02% NaN_3), and slowly heated to 100 °C, left for 1 h at that temperature while shaking and debranched for 16 h at 40 °C with 10 units of isoamylase from *Pseudomonas sp*. The debranched samples were dialyzed (1000 MWCO) against R. O. water and freeze-dried.

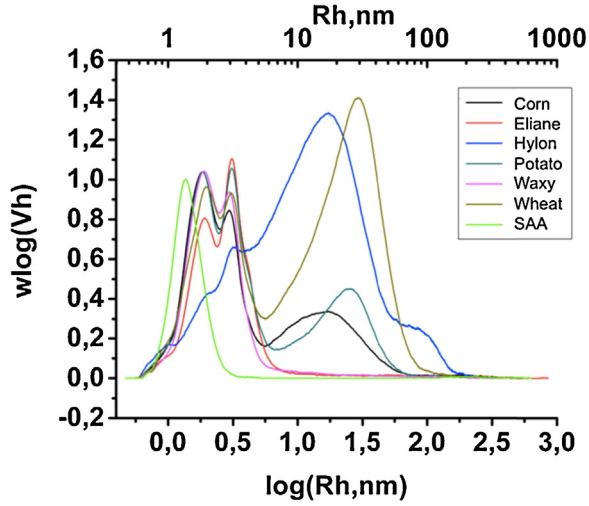

Fig. 1. SEC weight distributions $w(\log V_h)$ of the debranched starches.

2.3. SEC of the polysaccharides in DMSO/LiBr

The SEC system setup (Agilent Technologies 1260 Infinity) from PSS (Mainz, Germany) consisted of an isocratic pump, auto sampler without temperature regulation, an online degasser, an inline 0.2 μm filter, a refractive index detector (G1362A 1260 RID Agilent Technologies), viscometer (ETA-2010 PSS, Mainz), and MALLS (SLD 7000 PSS, Mainz). WinGPC Unity software (PSS, Mainz) was used for data processing. The samples (100 μL) were injected with a flow rate of 0.5 mL min^{-1} into a PFG guard-column and three PFG SEC columns 100, 300, and 4000, which were also purchased from PSS. The columns were held at 80 °C, and the detectors were held at 65 °C (Visco) and 45 °C (RI). A standard pullulan kit (PSS, Mainz, Germany) with molecular weights from 342 to 805 000 Da was used to generate a universal calibration curve, in order to determine the hydrodynamic volume from the elution volume.

The specific RI increment value, dn/dc for the well-defined linear polysaccharides in this system was taken to be the same as amylose, 0.0689 mL g^{-1}, since they are debranched. The samples (2 g L^{-1}) were mixed in DMSO/LiBr (0.05 M) for 3 h at 80 °C and overnight at room temperature by a thermo shaker with 350 rph. All samples were filtered through 0.45 μm filters. Standards were dissolved in the same eluent at room temperature at 2 g L^{-1} concentration. The Mark–Houwink parameters for pullulan in this eluent at 80 °C were measured by PSS and are $K = 2.424 \times 10^{-4}$ dL g^{-1} and $\alpha = 0.68$. The specific RI increment value dn/dc was also measured by PSS and is 0.072 mL g^{-1} (private communication with PSS).

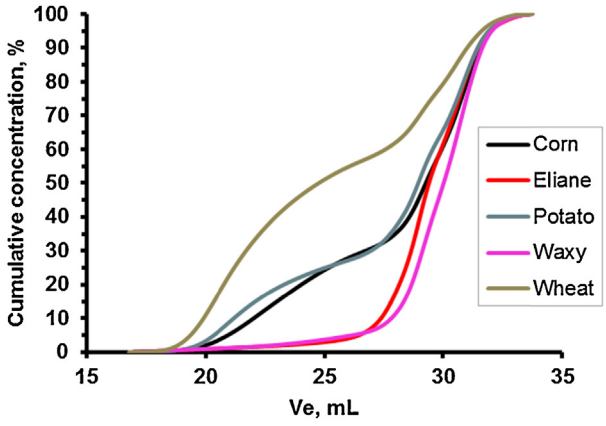

Fig. 2. Cumulative concentration distributions of the debranched starches.

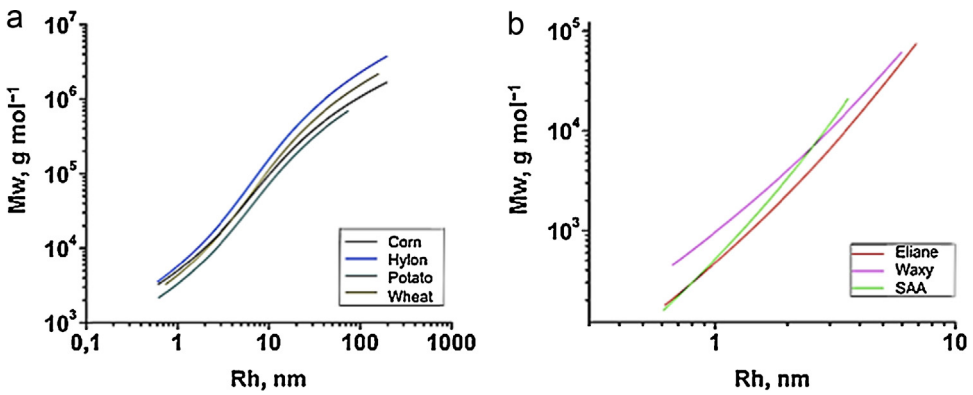

Fig. 3. Molar masses ((a) LS and (b) viscometer) versus R_h of the debranched starches.

3. Results and discussion

A wide range of different starches with various amylose contents were analyzed (regular starches – potato, wheat or corn contain normally around 25% of amylose, Hylon VII contains 70% of amylose, while the amylose content of Waxy starches is often less than 1%.). To compare with our previous results one synthetic amylopectin analog (SAA, reaction time 6 h, G-1-P/G-7 = 500) was included and subjected to the same treatment and analysis as natural starches.

The starches were debranched by isoamylase for 16 h and after purification analyzed by SEC with multi detection in DMSO/LiBr.

The distributions of the debranched starches are shown in Fig. 1. As expected the distribution patterns of high amylopectin content starches (99% amylopectin-Eliane and Waxy corn) had a characteristic bimodal peak apportioned to the short amylopectin branches, and no amylose peak.

The bi-modal amylopectin fraction of the other starches and Eliane shows that Eliane has a higher ratio of long versus short chains. The SAA had one characteristic peak in the area of short branches, which confirmed the conclusions made in our previous research. It was noticed that if the ratio between G-1-P and maltoheptaose is higher than 400, branching pattern becomes similar to glycogen branching pattern, with many short branches and the most dominant branch length of around 7 glucose units (Ciric et al., 2012).

For the short amylopectin branches of the normal starches a bimodal peak with R_h at $\log(R_h, \text{nm}) < 0.7$ (corresponding approximately to DP < 100) is clearly visible, see Fig. 1. The separation from the amylose long chains peak ($\log(R_h, \text{nm}) > 0.7$) is not 100% complete but clearly observable.

From these distributions, it is possible to calculate the ratio between amylose and amylopectin in the normal starches by comparing the areas under the curves (AUC) of the amylose peak to the total AUC of both amylopectin and amylose peaks if the peaks are separated or by simply checking a cumulative concentration distribution (corn and potato starch 30% amylose; Waxy and Eliane 5% amylose and wheat starch 55%, see Fig. 2).

Overlap between two areas is visible for Hylon, a high amylose content starch, which can be considered normal for this type of hybrid starches when debranched (Vilaplana et al., 2012). They consist mostly of amylose, therefore have much smaller molar masses than normal starches. Consequently it is worthless to attempt to separate the two areas with 1D chromatography, since the distribution of branches become wide and merges with amylose chains distribution.

The only unexpected result was obtained in the case of wheat starch. It appeared that it has a higher amylose content than expected (ca. 55% while below 30% is expected). As the separation between the amylopectin and amylose branch peaks is very good we suppose that in this case debranching was insufficient and/or low molar mass amylopectin was lost during purification.

Fig. 3 represents the molar masses versus R_h of the debranched starches. For the molar mass distributions, in which the molecular size of one part of the debranched starches was too small for MALLS detection (in the case of Eliane, Waxy and the SAA – high amylopectin content) we represented the data collected via viscometer detection using universal calibration based on pullulan standards (Ciric et al., 2012).

As expected, when amylopectin is debranched, the molar mass decreases drastically and the molar masses of the linear amylose parts reach much higher values since amylose has almost no branches which will be cut from the main chain by isoamylase. When compared with literature we see a very good agreement in both figures for the molar mass and R_h of the linear chains (Rolland-Sabaté, Mendez-Montealvo, & Colonna, 2008). For instance, R_h of 5 nm and 20 nm correspond to molar masses of 2.5×10^4 and $2–3 \times 10^5$, respectively. On the other hand molar masses of the branched polysaccharides with the same R_h are expected to be at least one magnitude bigger (Ciric et al., 2012). A slight difference in molar mass for the same R_h is visible for the different starches. It is known that isoamylase is not able to debranch amylose completely (Hizukuri, Takeda, Yasuda, & Suzuki, 1981) and therefore hylon appears to be the most branched in the amylose area after debranching.

4. Conclusions

With these analyses we showed that SEC with multi detection, PFG (modified silica) colums and DMSO/LiBr can be used for the characterization of debranched natural starches. In future work PFG columns with different particle dimensions should be used, in order to investigate the whole-molecule size distribution and a 2D analysis.

The amylose content can easily be determined. Additionally, the agreement for the relation size – molar mass of debranched parts was in a good agreement with literature.

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