Polymer 55 (2014) 6271–6277

Contents lists available at ScienceDirect

Polymer

journal homepage: www.elsevier.com/locate/polymer

Characterization of enzymatically synthesized amylopectin analogs via asymmetrical flow field flow fractionation

Jelena Ciric [a, b], Agnès Rolland-Sabaté [c], Sophie Guilois [c], 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
[c] UR1268 Biopolymères Interactions Assemblages, INRA, F-44300 Nantes, France

ARTICLE INFO

Article history:
Received 7 August 2014
Received in revised form
27 September 2014
Accepted 29 September 2014
Available online 5 October 2014

Keywords:
Asymmetrical flow field flow fractionation
Amylopectin
Enzymatic polymerization

ABSTRACT

Asymmetrical flow field flow fractionation (AF4), when coupled with multi-angle laser light scattering (MALLS), is a very powerful technique for determination of the macromolecular structure of high molar mass (branched) polysaccharides. AF4 is a size fractionation technique just as size exclusion chromatography (SEC), nevertheless can overcome some crucial problems found in SEC analysis especially in starch like structures. This paper describes a detailed investigation of the macromolecular structure of two groups of well-defined synthetic amylopectin analogs – synthesized via an *in vitro* enzyme-catalyzed reaction using the enzymes phosphorylase *b* from rabbit muscle and *Deinococcus geothermalis* glycogen branching enzyme (Dg GBE). Size, molar mass distributions and structural data were studied by AF4 coupled with online quasi-elastic light scattering (QELS) and multi-angle light scattering (MALLS).

1. Introduction

The most popular and the most developed method for determination of the size distributions of starch and starch-like polymers is size-exclusion chromatography (SEC) [1]. However, for good separation of branched and large polysaccharides in SEC the used columns and their limitations can be an obstacle. Currently used columns have low exclusion limits for extremely large polysaccharides, and lead to interactions of polysaccharides with the column material - especially in water; shear scission, and inevitable band broadening [1,2]. A technique that can overcome these problems is asymmetrical flow field flow fractionation (AF4) that has already been used for the analysis of natural and synthetic branched polysaccharides [3–5]. The separation process is based on differences in the diffusion coefficients of the analyzed components (separation depends on the size and shape of particles and molecules) [7–9]. AF4 selectivity is based on the diffusion coefficient of the sample; hence the hydrodynamic radius (R_h) can be determined via the diffusion coefficient from the Stokes–Einstein equation.

$$R_h \equiv \frac{k_B T}{6\pi\eta D_T} \tag{1}$$

where D_T represents the translational diffusion coefficient, k_B Boltzman's constant, T the temperature, and η the viscosity of the solvent.

The retention ratio in the normal mode (small particles are eluted first) with constant crossflow according to the AF4 theory is [10]:

$$R = \frac{t_0}{t_{ri}} \approx \frac{6 D_i V_0}{F_c w^2} \tag{2}$$

where t_0 represents the void time (the time needed for the carrier solvent to pass from inlet to outlet in the channel), t_{ri} elution time, D_i diffusion coefficient of the i_{st} exponent slice, F_c is the crossflow rate, w the channel thickness, and V_0 the void volume (the geometric volume of the channel).

For samples with high size dispersities, like the enzymatically synthesized amylopectin analogs studied here, a good fractionation of the whole sample can be achieved using a crossflow gradient instead of a constant crossflow. For an exponential regression of a crossflow t_{ri} can be expressed as [4]:

* Corresponding author. University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands. Tel.: +31 50 3636867.
E-mail addresses: jelenaciricnl@gmail.com (J. Ciric), Agnes.Sabate@nantes.inra.fr (A. Rolland-Sabaté), sophie.guilois@nantes.inra.fr (S. Guilois), k.u.loos@rug.nl, katjaloos@web.de (K. Loos).

http://dx.doi.org/10.1016/j.polymer.2014.09.059
0032-3861/

$$t_{ri} = t_{start} + (t_{end} - t_{start})\left\{1 - \exp\left[-\frac{\frac{t_0 w^2 F_{c_{start}}}{6DV_0} - t_{start}}{t_{end} - t_{start}}\right]\right\} \quad (3)$$

where t_{start} is the time corresponding to the start and t_{end} the time corresponding to the end of the gradient.

Size distributions can be obtained by determining the relationship between D_T and t_{ri} using established equations for methods with constant crossflow [11], and their modifications for gradient crossflow [12,13]. If possible, R_h can be determined online by means of quasi-elastic light scattering (QELS), providing experimental size distributions.

In our previous paper we have solved parts of the molecular mechanism of the enzymatic polymerization of amylopectin analogs, see Fig. 1. These analogs were synthesized *in vitro* using the enzymes phosphorylase *b* and *Deinococcus geothermalis* glycogen branching enzyme (Dg GBE). The synthesized analogs had tunable degree of branching, obtained by regulation of the reaction time, and tunable degree of polymerization obtained by regulation of the ratio between the monomer glucose-1-phosphate (G1P) and the primer maltoheptaose (G7) (i.e. regulation of monomer concentration) [6]. We have seen that with an increase of the reaction time (average degree of branching detected by ^{1}H NMR), an unexpected constant increase of the molecular mass is detected by SEC after all available monomer is consumed in the reaction. We explained this anomaly by the hypothesis that some chains serve mostly as branch donors and some as acceptors during the one-pot enzymatic synthesis. Furthermore, we have noticed that with an increase of the monomer concentration (degree of polymerization, followed by released inorganic phosphate from the used monomer G1P), an unexpected constant decrease of the molecular mass occurs. This atypical behavior was explained by hindered diffusion which changes the preferences of the enzymes and the fact that some of the cleaved short oligosaccharides serve as primers instead of becoming branches [6].

For this reason, in order to verify the conclusions previously made based on the analysis, we compare and verify them by analyzing the same enzymatically synthesized amylopectin analogs with AF4.

The system used for the separation and characterization of amylopectin analogs was an AF4-MALLS-QELS setup. We successfully determined the macromolecular characteristics and the structural information of the branched polysaccharides and concluded to have highly branched glycogen-like particles. Here we show that AF4 can superbly be used for the fractionation of highly branched polysaccharides with various sizes and degrees of branching. Moreover, we confirm all the previously made assumptions based on SEC analysis concerning the enzymatic synthesis used in this work. By comparing, fulfilling, correcting and combining the results from two powerful techniques such as SEC and AF4 with multi detection, we were able to show how important the establishment of improved characterization protocols for branched polysaccharides is.

2. Experimental

2.1. Materials and methods

All chemicals used for the synthesis and AF4 (glucose-1-phosphate (G-1-P), tris(hydroxymethyl)aminomethane (Tris), dithiothreitol (DTT), adenosine monophosphate (AMP), phosphorylase *b*, sulfuric acid, NaN_3, Orcinol) were purchased from Sigma–Aldrich and used without further purification. Glycogen branching enzyme from *D. geothermalis* (Dg GBE) was kindly provided by R.J. Leemhuis and L. Dijkhuizen, whereas maltoheptaose (G-7) was synthesized as explained elsewhere [14]. The water used for analysis and sample preparation was produced by a RiOsTM and Synergy purification system (Millipore, Bedford, MA, USA).

2.2. Synthesis of well-defined branched polysaccharides [6]

G-1-P was dissolved in Tris buffer (100 mM, pH 6.7, 0.02% NaN_3) 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

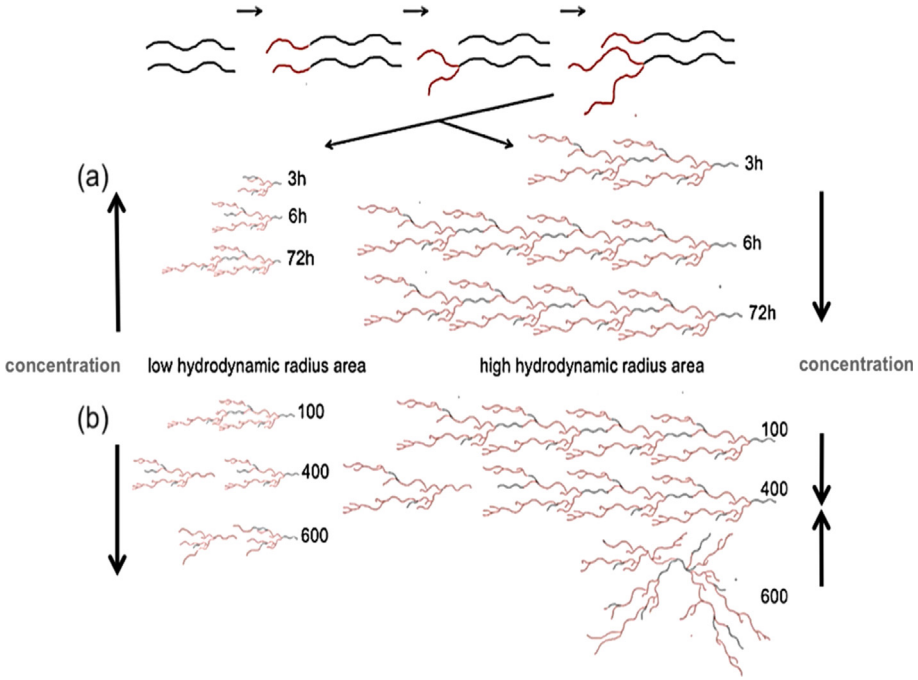

Fig. 1. Proposed mechanism of the *in vitro* enzymatic synthesis of branched polysaccharides using phosphorylase *b* and Dg GBE. a) represents the first group of samples – reaction time was varied. b) represents the second group of samples – concentration was varied.