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Synthesis of branched polysaccharides with tunable degree of branching

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ABSTRACT

An in vitro enzyme-catalyzed tandem reaction using the enzymes phosphorylase *b* from rabbit muscle and Deinococcus geothermalis glycogen branching enzyme (Dg GBE) to obtain branched polyglucans with tunable degree of branching (2% ÷ 13%) is presented. The tunable degree of branching is obtained by varying the reaction conditions such as pH value, the choice of reducing agent and its concentration and reaction time. Linear amylose is formed by the phosphorylase-catalyzed propagation of glucose-1-phosphate while Dg GBE introduces branching points on the α-(1 → 6) position by relocating short oligosaccharide chains. Our results show that the best way to obtain different degrees of branching with this set of enzymes is by regulation of the reaction time.

1. Introduction

Branched carbohydrates such as amylopectin differ to a large extent from their linear analogues, for instance they show differences in solubility, or rheological and mechanical properties. Many of the properties are necessary for the food and non-food applications of starch and therefore the meticulous characterization of the molecular weight, the type and the degree of branching of such materials is of great importance. However, good non-destructive characterization techniques for branched polysaccharides are rare (Viliaplana & Gilbert, 2010).

In order to establish an improved protocol for the characterization of branched polysaccharides well-defined branched standards are required. Standards are materials that provide a reference to determine unknown concentrations or to calibrate analytical instruments.

In general the molecular size is not only dependent on the molecular weight but also on the degree of branching as branching reduces the molecular size (Podzimek, Vlcek, & Johann, 2001; Pollock & Ktatz, 1980, chap. 2).

The organic synthesis of well-defined (branched) polysaccharides such as chemical glycosylation is rather time-consuming and complicated; however, when enzymes are introduced as biocatalysts, the desired carbohydrates can be obtained easily (Kaper et al., 2005; Kobayashi, Uyama, & Kimura, 2001; Kralj et al., 2004) Therefore, enzymatic polymerization (Loos, 2010) can be utilized for the synthesis of branched polysaccharides as standards for improved characterization methods. For example, amylopectin analogue, hyper-branched amylose can be synthesized in vitro by combined action of phosphorylase (EC 2.4.1.1) and glycogen branching enzyme (GBE) (EC 2.4.1.18) (Fujii et al., 2003; Yanase, Takaha, & Kuriki, 2006). It was reported that branched polyglucans with the degree of branching of 11% can be synthesized by the tandem action of potato phosphorylase and *Deinococcus geothermalis* glycogen branching enzyme (Dg GBE) (van der Vlist et al., 2008).

However the reported synthesis has a couple of drawbacks. The potato phosphorylase formulation is not very pure and the resulting amylopectin analoga are contaminated with other carbohydrates. Furthermore it was not possible to alter the degree of branching by using potato phosphorylase. It is known that phosphorylase *b* from rabbit muscle (GPb) has more than 75% of the same active-site residues as potato phosphorylase and they have many resemblances in their catalytic properties (Fukui, Shimomura, & Nakano, 1982). GPb's action is well known in the literature and some GPbs are commercially available enzymes. We therefore decided to use GPb for the polymerization of amylopectin analoga.

In cells, phoshorylase releases glucose-1-phosphate (G-1-P) from the non-reducing end of α-(1 → 4)-glucan chains. This reaction is reversible, hence when used in vitro with high excess of G-1-P, phosphorylases catalyze the addition of glucose units to the chain and inorganic phosphate is released. It is well known that this synthesis requires the presence of a recognition unit suitable to start the polymerization which is a co-substrate such as starch, glycogen or oligosaccharides (Cori & Cori, 1939, 1940; Suganuma, Kitazono, Yoshinaga, Fujimoto, & Nagahama, 1991). The enzyme

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activity is controlled by the internal conversion between alternative structural states of the enzyme (Barford, Hu, & Johnson, 1991). GPb is the non-phosphorylated form of the enzyme and is activated by adenosine 5′-monophosphate (AMP) (Ho & Wang, 1973).

In nature, glycogens' side chains are introduced by glycogen branching enzyme. This enzyme is responsible for α-(1 → 6) branch points formation, by cutting α-(1 → 4) glycosidic linkage in the donor chain and transferring oligosaccharide from the nonreducing end to the α-(1 → 6) position (Boyer & Preiss, 1977). It was previously shown that Dg GBE can be used to introduce a high degree of branching with an unusual side-chain distribution (Palomo, Kralj, van der Maarel, & Dijkhuizen, 2009).

Phosphorylase and branching enzyme together make the synthesis of branched polysaccharides via a one-pot synthesis possible. Phosphorylase will polymerize linear amylose and the branching enzyme will introduce the branching points. Newly introduced branching points, later on serve as new "starters" for the phosphorylase. The tandem polymerization using the two enzymes can be seen in Scheme 1.

Enzymatic activity can be affected by many factors such as temperature, pH value, water activity, ionic strength, chemicals such as reducing agents, etc. For example, the thiol groups of some enzymes are voluntarily oxidized in air to disulfides (Clealand, 1964). Reducing agents such as glutathione (GSH), dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP) are often added to maintain these groups in the reduced state (Han & Han, 1994).

By using GPb and Dg GBE as phosphorylase and branching enzyme respectively we succeeded in obtaining amylopectin analoga in high purity. By varying the pH, the reaction time and the type and concentration of the reducing agent the molecular features of the branched polysaccharides (such as the degree of branching) could be adjusted opening the possibility to synthesize standards for the further development of characterization techniques.

2. Materials and methods

All reagents and enzymes, if not mentioned otherwise, were purchased from Sigma–Aldrich and were used without further purification. Dg GBE was produced as described in literature (Palomo, Kralj, van der Maarel, & Dijkhuizen, 2009). Potato phosphorylase was isolated and G-7 was synthesized as reported elsewhere (van der Vlist et al., 2008). ^{1}H NMR spectra were recorded on a Varian Inova 400 MHz spectrometer at 50 °C. 2,2-Dimethyl-2-silapentane-5-sulfonic acid (DSS) was used as an external standard. 10–20 mg samples were dissolved in 700 μL D_2O. UV–vis measurements were recorded with a PYE Unicam sp8 200 spectrophotometer. Attenuated Total Reflection-Fourier Infrared (ATR FT-IR) measurements were carried out on a Bruker IFS88 FT-IR spectrometer equipped with a MCT-A detector, at a resolution of 4 cm^{-1} with an average of 50 scans.

2.1. Determination of the degree of polymerization

The degree of polymerization was followed via colorimetric measurement of the released inorganic phosphate. Inorganic phosphate analysis was performed according to literature procedures (van der Vlist, 2011, chap. 2).

2.2. Determination of the degree of branching

The average degree of branching of the synthesized polysaccharides was determined with ^{1}H NMR. The anomeric protons of α-(1 → 4) and α-(1 → 6) linked sugar residues give separate signals at 5.42 and 5.01 ppm, respectively. The average degree of branching can be determined by the use of the ratio between the relative areas below the signals (Nilsson, Bergquist, Nilsson, & Gorton, 1996). The hydroxylic protons of the samples were exchanged in D_2O. In order to ensure the complete relaxation of the protons, a pause of 10 s between pulses was taken.

2.3. Synthesis of maltoheptaose

Maltoheptaose was synthesized via ring opening of β-cyclodextrin according to literature (van der Vlist et al., 2008).

2.4. Synthesis of branched polyglucans

In order to establish a library of polyglucans with various degree of branching the methods described in literature by van der Vlist et al. (2008) and Liu, Castro, and Gilbert (2011) were adjusted accordingly. Different buffers (tris(hydroxymethyl)aminomethane (Tris) 100 mM, pH 7, 0.02% NaN_3; 3-(N-morpholino)propanesulfonic acid (MOPS) 50 mM, pH 7, 0.02% NaN_3) and reducing agents were used (DTT, GSH, TCEP). pH value of the reaction mixture, reducing agent concentration and the reaction time were varied whereas the concentration of G-1-P, G-7, AMP, GPb and Dg GBE were maintained constant if not stated differently. Excess G-1-P, AMP and reducing agents were removed by means of dialysis. Subsequently the samples were freeze-dried.

2.5. Purity of the branched polyglucans and synergetic action of the enzymes

A mixture consisting of G-7 (0.3 mM), G-1-P (25 mM), GPb (0, 0.33 and 0.33 μM), Dg GBE (50, 0 and 50 U mL^{-1}), AMP (3.5 mM) and DTT (1.3 mM) dissolved in 2 mL buffer (Tris 100 mM, pH 7, 0.02% NaN3) was incubated for 72 h at 37 °C.

2.6. Effects of the reducing agent on the degree of branching

A mixture consisting of G-7 (0.3 mM), G-1-P (50 mM), GPb (0.42 and 0.25 μM), Dg GBE (50 and 50 U mL^{-1}), AMP (3.5 mM) and DTT (0, 0.6, 1.3, 2.5, 5 mM) or TCEP (0, 0.6, 1.3, 2.5, 5 mM) or GSH (0, 0.6, 1.3, 2.5, 5 mM) dissolved in 2 mL buffer (Tris 100 mM, pH 7, 0.02% NaN3) was incubated for 72 h at 37 °C.

2.7. Effects of pH on the degree of branching

A mixture consisting of G-7 (0.3 mM), G-1-P (50 mM), GPb (0.25 μM), Dg GBE (50 U mL^{-1}), AMP (3.5 mM) and DTT (1.3 mM) dissolved in 2 mL buffer (Tris 100 mM, pH 6, 6.5, 6.7, 7, 7.5, 8, 8.5 and 9 0.02% NaN_3) was incubated for 72 h at 37 °C.

2.8. Effects of time on the degree of branching

A mixture consisting of G-7 (0.3 mM), G-1-P (50 mM), GPb (0.42 μM), Dg GBE (50 U mL^{-1}), AMP (3.5 mM) and DTT (1.3 mM) dissolved in 2 mL buffer (Tris 100 mM, pH 7, 0.02% NaN_3) was incubated for 1, 2, 3, 4, 5, 6, 8, 24, 48, 72 and 100 h at 37 °C.

3. Results and discussion

3.1. Purity of the branched polyglucans and synergetic action of the enzymes

During our previous work, branched polyglucans were successfully synthesized with potato phosphorylase in combination with Dg GBE (van der Vlist, 2011), however these polyglucans were not pure enough for the present research. Since potato phosphorylase was isolated from potatoes the synthesized polymers had