Carbohydrate Polymers 93 (2013) 31–37

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

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

Synthesis of branched polysaccharides with tunable degree of branching

Jelena Ciric, Katja Loos*

Department of Polymer Chemistry, Zernike Institute for Advanced Materials, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

ARTICLE INFO

Article history:
Received 17 November 2011
Received in revised form 29 February 2012
Accepted 5 April 2012
Available online 16 April 2012

Keywords:
Degree of branching
Glycogen branching enzyme
Phosphorylase
Polysaccharides

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

* Corresponding author. Tel.: +31 50 3636867; fax: +31 50 3634400.
E-mail address: k.u.loos@rug.nl (K. Loos).

0144-8617/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2012.04.008

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.

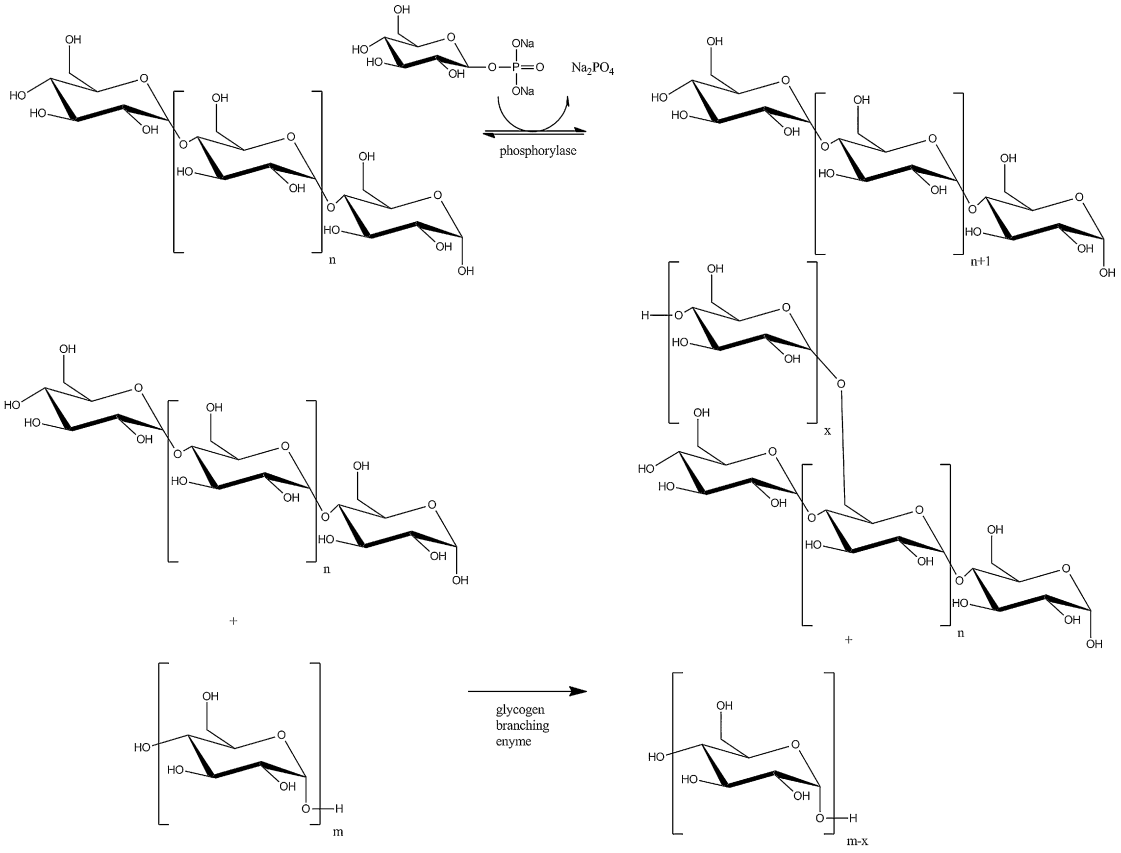
2.8. Effects of time on the degree of branching

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



Scheme 1. Tandem enzymatic polymerization: the actions of phoshorylase and glycogen branching enzyme, respectively.

impurities from the tubers (starch, proteins and other high molecular weight compounds). Moreover, when dissolved in water, the solution was never completely clear and colorless but opaque and yellowish due to the additional compounds from potato. Additionally it seemed to be not possible to change the degree of branching with this polymerization. As standards for improved characterization technique require the highest possible purity and solubility in the applied solvent, likewise the variety in the degree of branching, the synthesis required a modification.

An interesting publication from Liu, Castro, and Gilbert (2011) provoked us to combine Dg GBE with GPb instead of potato phosphorylase, even though GPb needs an activator and reducing agent (AMP and DTT respectively) and potato phosphorylase does not. After adjusting the conditions, the enzymatic tandem reaction with GPb and Dg GBE gave pure, water-soluble polysaccharides, the products dissolved in water as clear solution, which was also verified by the improved quality of ^{1}H NMR spectra and ATR FT-IR measurements. Fig. 1 illustrates ATR FT-IR spectra of pure G-7 (A), the polymer synthesized with GPb as the catalyst (B) and the polymer synthesized with potato phosphorylase as the catalyst (C). On one hand the spectrum (B) is the same as the spectrum (A), which confirms the purity of the polysaccharide. On the other hand the spectrum (C) differs from the spectrum (A) by the peak in the amide I′ components band (1700–1600 cm^{-1}) characteristic for the enzyme (Byler & Susi, 1986; Griessler, D'Auria, Tanfani, & Nidetzky, 2000), which confirms contamination of the polymer with the enzyme.

It has been shown that the growth of the polymer is linear until the equilibrium state is reached, and that the tandem reaction of potato phosphorylase with Dg GBE follows the same kinetics as the reaction catalyzed solely with potato phosphorylase (van der Vlist et al., 2008).

When GPb was introduced instead of potato phosphorylase it became obvious that the initial stage of the conversion is much faster for the tandem reaction; and that the conversion in general is higher (see Fig. 2). It was already reported in literature that muscle phosphorylases act more on branched acceptors than on linear ones (Cori & Cori, 1936; Goldemberg, 1962) which explains this higher conversion for the tandem polymerization. The reaction mixture containing only Dg GBE was used as a blank reaction showing that DgGBE alone does not produce any linear or

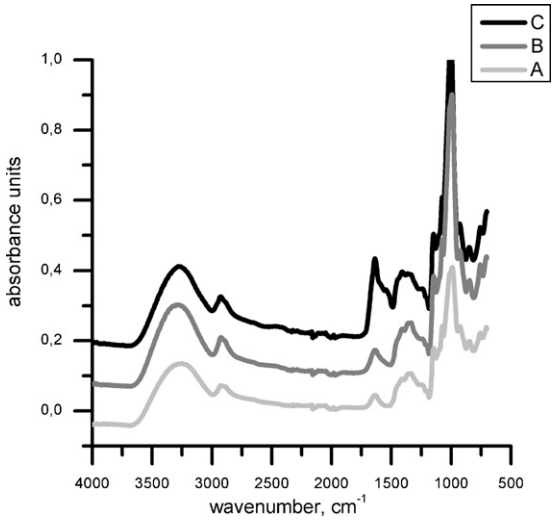


Fig. 1. ATR FT-IR spectra of (A) pure G7, (B) the polymer synthesized with GPb as the catalyst and (C) the polymer synthesized with potato phosphorylase as the catalyst.

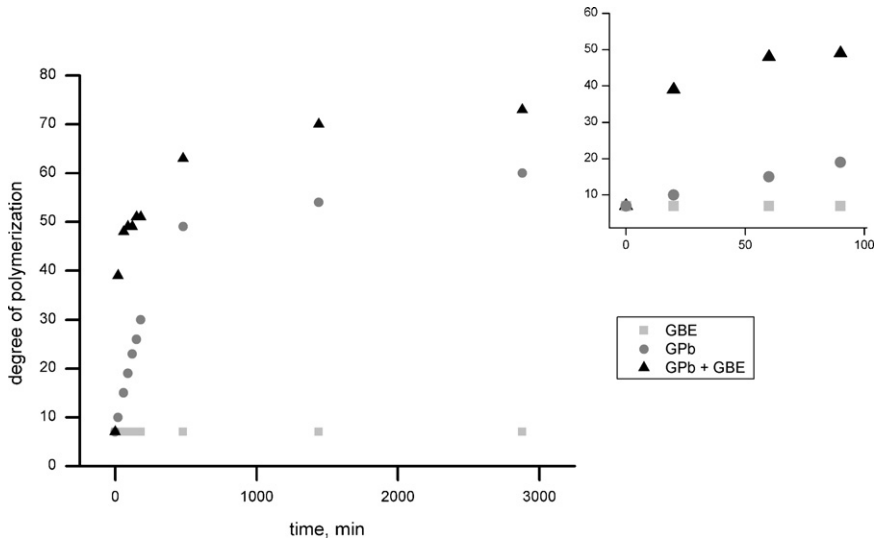


Fig. 2. Kinetics of the GPb-catalyzed polymerization and the tandem polymerization.

branched polysaccharides which is in accordance with the reaction mechanism of this enzyme (Palomo et al., 2011).

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

Proteins are in a reducing environment when found in nature; hence sulfhydryl groups of enzymes are preserved in their reduced form. When dissolved under laboratory conditions, reducing agents must be added to imitate in vivo conditions in order to maintain the enzyme's function (Getz, Xiao, Chakrabarty, Cooke, & Selvin, 1999). Three common reducing agents in biochemistry were screened and the products of the enzymatic tandem polymerization were compared in order to see whether the degree of branching can be regulated by varying the concentration and the type of the reducing agent. A reference reaction was performed, without any reducing agent. The concentration of each reducing agent was varied from 0.6 mM to 5 mM for all agents. Each of the agents follows a different trend, which can be seen in Fig. 3. DTT has a tendency to increase the degree of branching in very small quantities reaching a maximum at 0.6 mM, with a continuous decrease of the degree of branching at higher agent concentrations (Fig. 3(A)). TCEP, which is the strongest reducing agent, has a constant trend for this range of concentrations by increasing the degree of branching to the same value with an exception for the concentration of 2.5 mM (Fig. 3(B)). The only natural antioxidant GSH continuously increases the degree of branching with an increase in concentration (Fig. 3(C)).

During the experiments we also noticed that the reducing agents have an impact on the degree of polymerization. The addition of GSH, for both quantities of GPb, increases the degree of polymerization from 103 to 133. On the other hand the addition of TCEP reduces the degree of polymerization from 103 to 84 (Supplementary data Table S1).

Our current work shows that the tandem reaction can be performed successfully without any reducing agent; however the type and the concentration of the reducing agent have an impact on the degree of polymerization and especially on the degree of branching. A small amount of the reducing agent increases the degree of branching. We assume that the partial protonation of the Dg GBE, together with the possibility of the protonation of the active site, results in the increase of the degree of branching as outlined below. In this experiment it becomes obvious that the degree of branching can be regulated by the amount of reducing agent. Moreover, it can be clearly seen that each of the selected agents follows a different trend for the dependence of the degree of branching and the agent's concentration, due to the different agent's properties. The phenomenon, that different reducing agents follow different trends is familiar in literature (Mesecke et al., 2005). In the following work we use only DTT as the reducing agent, since that is the most commonly used one. Additionally we use only one concentration of GPb since there is no drastic difference in the results for different concentrations.

3.3. Effects of pH on the degree of branching

It is well known that the enzymes are sensitive to the pH conditions of the solution. In order to choose the best buffer for further studies different buffers were used (Tris 100 mM, pH 7, 7.5 and 8, 0.02% NaN_3; MOPS 50 mM, pH 7, 0.02% NaN_3) for the synthesis of branched polyglucans via the one-pot enzymatic tandem polymerization. The results were polymers of similar degree of polymerization and the same degree of branching for both buffers when compared at equal pH. In literature it was reported, that Tris buffer is best used when GPb from rabbit liver is used as an enzyme (Zea & Pohl, 2004). Consequently, Tris buffer was our choice for the future, since it can be used for a broader range of pH values.

The tandem polymerization reaction was effectively performed with potato phosphorylase at a pH of 7.0 at 37 °C according to literature (van der Vlist et al., 2008). However, the optimum reaction conditions of GPb and Dg GBE are pH 6.9 to 7.4/38 °C (Brandt, Cafwlong, & Lee, 1975) and pH 8.0/34 °C, respectively. By varying the pH value we noticed that by an increase of the pH value, starting with the lowest value of 6, the degree of branching increases and reaches the highest value of around 13% for a pH of 6.7 and afterwards starts to decrease continuously until the highest analyzed value of pH 9.

The only decrease in degree of polymerization is observed between pH values of 7.5 and 8.5, whereas for the other values it stays constant (around 94) at equal reaction conditions (Supplementary data Table S2). It becomes obvious that the degree of branching changes drastically with changing pH while the degree of polymerization in most cases stays the same. That brings us to the conclusion that Dg GBE is very pH sensitive whereas GPb is not. This is in accordance with literature – GPb is pH insensitivity above pH 6.4 (Feldmann & Hull, 1977). This effect allows us to synthesize branched polysaccharides with tunable degree of branching. The

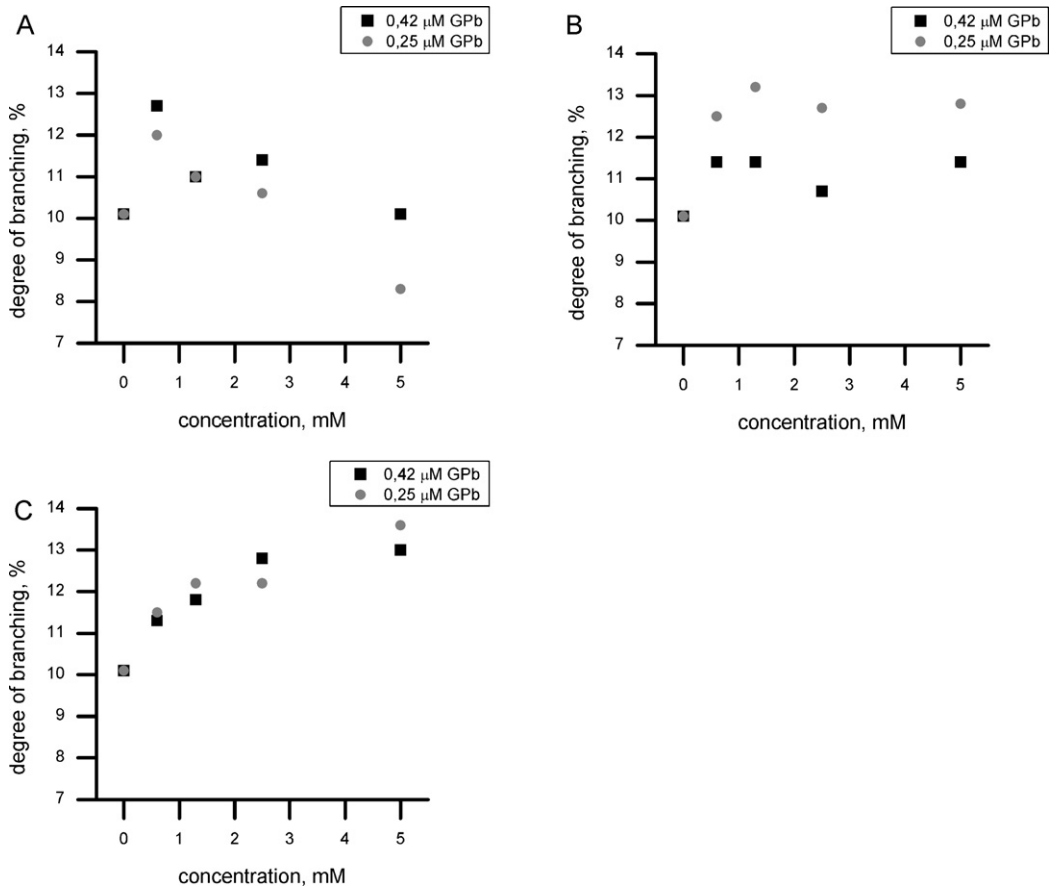


Fig. 3. The degree of branching of polysaccharides synthetized with different reducing agents with different reducing agent concentrations. (A) DTT (B) TCEP (C) GSH.

obtained degrees of branching at the different pH values after 22 h of reaction time at 37 °C are depicted in Fig. 4.

Our method enables tuning of the degree of branching (8–13%) and reaching higher degrees of branching than previously reported. The pH dependence of the degree of branching (activation of the Dg GBE) with addition of reducing agent is possible evidence of partial protonation of the active site of the Dg GBE. This is similar to the investigation of another group of enzymes reported in literature (Olsen et al., 1992). Furthermore, with the newly introduced enzyme, GPb, and a slight modification of pH values, addition of the enzyme activator and the reducing agent, branched polyglucans are synthesized with higher degree of branching than previously reported with potato phosphorylase (van der Vlist et al., 2008). This is possible due to the partial protonation of the Dg GBE, including the possibility of the protonation of the active site.

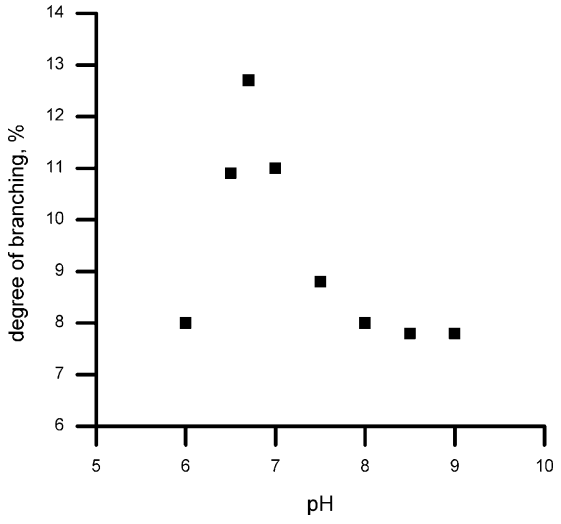


Fig. 4. The degree of branching of polysaccharides synthetized at different pH values.

3.4. Effects of time on the degree of branching

A satisfactory degree of polymerization is achieved after a short period of time, which provides the possibility to adjust the degree of branching via time regulation (see Table 1). This is due to the faster catalyzed polymerization of the linear chains by GPb compared to

Table 1
The degree of branching of polysaccharides acquired for different reaction time.

Time [h]	Ratio G1P/G7	Degree of polymerization[a]	Degree of branching[b] [%]
1	166.7	79	1.6
2	166.7	94	5
3	166.7	94	6
4	166.7	108	6.3
5	166.7	112	6.3
6	166.7	112	7.7
8	166.7	120	9
24	166.7	120	10
48	166.7	135	12.5
100	166.7	135	12.5
72	66.7	68	17.8

[a] Determined via the colorimetric measurement of the liberated inorganic phosphate.
[b] Determined via ^{1}H NMR spectroscopy.

the cleavage and transfer of short oligosaccharides by Dg GBE in the tandem reaction. Therefore, the reaction can simply be terminated at the required degree of polymerization and degree of branching, by heat treatment.

If a lower degree of branching is required at a higher degree of polymerization, it can be regulated by adjusting the ratio of G-1-P and G7 (van der Vlist et al., 2008). However, it was noticed that the degree of branching decreases with the increase in degree of polymerization for the same reaction conditions and different ratios of G-1-P and G-7. This is possibly due to steric hindrance, since the increase of concentration of Dg GBE does not increase the degree of branching. Too large number of molecules could prevent enzymes to reach the polysaccharide and catalyze both further polymerization and branching. In order to determine this further investigations are currently under way.

An exceptionally high degree of branching (around 18%) was only obtained for the smallest tested ratio of G-1-P and G-7 and the degree of polymerization of 68, other values varied from 11% to 13% (Supplementary data Fig. S2).

It is clear from the data at Table 1 that the degree of branching increases with time when GPb is used in the tandem polymerization, in comparison to the constant degree of branching throughout the whole reaction time when potato phosphorylase is used (van der Vlist, 2011). This is due to GPbs affinity towards branched polysaccharides (Goldemberg, 1962; Cori & Cori, 1936). The linear polymerization catalyzed by GPb is faster than the branching catalyzed by Dg GBE which means that time is the best mean to tune the degree of polymerization.

Therefore, it is possible to synthesize a wide variety of polysaccharides with different degrees of branching and polymerization.

4. Conclusions

By an enzymatic tandem polymerization using GPb and Dg GBE various branched polysaccharides with tunable degree of branching and polymerization were polymerized.

During the kinetics analyses we concluded that potato phosphorylase and GPb have different affinities towards branched polysaccharides in comparison to linear ones. The tandem enzymatic polymerization is robust enough to perform without addition of any reducing agents; nevertheless both the degree of polymerization and the degree of branching appeared to be dependent on reducing agent.

Different pH values of the reaction mixtures, different reaction times, diverse reducing agents and their concentrations were tested and it was shown that products with different branching properties can be obtained by varying those parameters. It was shown that the best way to obtain different degrees of branching was by regulating the reaction time. The obtained polymers are pure and easily dissolve in water, which facilitates further analysis.

Acknowledgments

This research was supported by the Dutch Polymer Institute under Project no. 727.

The authors would like to thank Prof. Dr. L. Dijkhuizen and Dr. R.J. Leemhuis for kindly providing Dg GBE and Dr. Dusan Popov-Celeketic for constructive suggestions.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2012.04.008.

References

Barford, D., Hu, S.-H., & Johnson, L. N. (1991). Structural mechanism for glycogen phosphorylase control by phosphorylation and AMP. *Journal of Molecular Biology*, *218*, 233–260.

Boyer, C., & Preiss, J. (1977). Biosynthesis of bacterial glycogen. purification and properties of the *Escherichia coli* B α-1,4-glucan:α-1,4-glucan 6-glycosyltransferase. *Biochemistry*, *16*, 3693–3699.

Brandt, H., Cafwlong, Z. L., & Lee, E. C. (1975). Purification and properties of rabbit liver phosphorylase phosphatase. *Journal of Biological Chemistry*, *20*, 8038–8044.

Byler, D. M., & Susi, H. (1986). Examination of the secondary structure of proteins by deconvolved FTIR spectra. *Biopolymers*, *25*, 469–487.

Clealand, W. W. (1964). Dithiothreitol, a new protective reagent for SH groups. *Biochemistry*, *3*, 480–482.

Cori, G. T., & Cori, C. F. (1936). An unusual case of esterification in muscle. *Journal of Biological Chemistry*, *116*, 129–132.

Cori, G. T., & Cori, C. F. (1939). The activating effect of glycogen on the enzymatic synthesis of glycogen from glucose-1-phosphate. *Journal of Biological Chemistry*, *131*, 397.

Cori, G. T., & Cori, C. F. (1940). The kinetics of the enzymatic synthesis of glycogen from glucose-1-phosphate. *Journal of Biological Chemistry*, *135*, 733.

Feldmann, K., & Hull, W. E. (1977). ^{31}P nuclear magnetic resonance studies of glycogen phosphorylase from rabbit skeletal muscle: Ionization states of pyridoxal 5′-phosphate. *Biochemistry*, *74*, 856–860.

Fujii, K., Takata, H., Yanase, M., Terada, Y., Ohdan, K., Takaha, T., et al. (2003). Bioengineering and application of novel glucose polymers. *Biocatalysis and Biotransformation*, *21*, 167–172.

Fukui, T., Shimomura, S., & Nakano, K. (1982). Potato and rabbit muscle phosphorylases: Comparative studies on the structure, function and regulation of regulatory and nonregulatory enzymes. *Molecular and Cellular Biochemistry*, *42*, 129–144.

Getz, E. B., Xiao, M., Chakrabarty, T., Cooke, R., & Selvin, P. R. (1999). A Comparison between the sulfhydryl reductants tris(2-carboxyethyl)phosphine and dithiothreitol for use in protein biochemistry. *Analytical Biochemistry*, *273*, 80.

Goldemberg, S. H. (1962). Specificity of uridine diphosphate glucose-glycogen glucosyltransferase. *Biochimica et Biophysica Acta*, *56*, 357–359.

Griessler, R., D'Auria, S., Tanfani, F., & Nidetzky, B. (2000). Thermal denaturation pathway of starch phosphorylase from *Corynebacterium callunae*: Oxyanion binding provides the glue that efficiently stabilizes the dimer structure of the protein. *Protein Science*, *9*

Han, J. C., & Han, G. Y. (1994). A procedure for quantitative determination of tris(2-carboxyethyl)phosphine, an odorless reducing agent more stable and effective than dithiothreitol. *Analytical Biochemistry*, *220*, 5–10.

Ho, H. C., & Wang, J. H. (1973). A calorimetric study of the interactions between phosphorylase b and its nucleotide activators. *Biochemistry*, *23*, 4750–4753.

Kaper, T., Talik, B., Ettema, T. J., Bos, H., van der Maarel, M. J. E. C., & Dijkhuizen, L. (2005). Amylomaltase of *Pyrobaculum aerophilum* IM2 produces thermoreversible starch gels. *Applied and Environmental Microbiology*, *71*, 5098–5106.

Kobayashi, S., Uyama, H., & Kimura, S. (2001). Enzymatic polymerization. *Chemical Reviews*, *101*, 3793–3818.

Kralj, S., van Geel-Schutten, G. H., Dondorff, M. M. G., Kirsanovs, S., van der Maarel, M. J. E. C., & Dijkhuizen, L. (2004). Glucan synthesis in the genus Lactobacillus: Isolation and characterization of glucansucrase genes, enzymes and glucan products from six different strains. *Microbiology*, *150*, 3681–3690.

Liu, W.-C., Castro, J. V., & Gilbert, R. G. (2011). Rate coefficients for enzyme-catalyzed reactions from molecular weight distributions. *Polymer*, *52*, 1490–1494.

Loos, K. (2010). *Biocatalysis in polymer chemistry*. Wiley-VCH.

Mesecke, N., Terziyska, N., Kozany, C., Baumann, F., Neupert, W., Hell, K., et al. (2005). A disulfide relay system in the intermembrane space of mitochondria that mediates protein import. *Cell*, *121*, 1059–1069.

Nilsson, G. S., Bergquist, K. E., Nilsson, U., & Gorton, L. (1996). Determination of the degree of branching in normal and amylopectin type potato starch with H-1-NMR spectroscopy – Improved resolution and two-dimensional spectroscopy. *Starch*, *48*.

Olsen, D. B., Hepburn, T. W., Lee, S.-L., Martin, B. M., Mariano, P. S., & Dunaway-Mariano, D. (1992). Investigation of the substrate binding and catalytic groups of the P—C bond cleaving enzyme, phosphonoacetaldehyde hydrolase. *Archives of Biochemistry and Biophysics*, *296*, 144–151.

Palomo, M., Kralj, S., van der Maarel, M. J. E. C., & Dijkhuizen, L. (2009). The unique branching patterns of deinococcus glycogen branching enzymes are determined by their N-terminal domains. *Applied and Environmental Microbiology*, *75*, 1355–1362.

Palomo, M., Pijning, T., Booiman, T., Dobruchowska, J., van der Vlist, J., Kralj, S., et al. (2011). Thermus thermophilus glycoside hydrolase family 57 branching enzyme. *Journal of Biological Chemistry*, *286*, 3520–3530.

Podzimek, S., Vlcek, T., & Johann, C. (2001). Characterization of branched polymers by size exclusion chromatography coupled with multiangle light scattering detector. I. Size exclusion chromatography elution behavior of branched polymers. *Journal of Applied Polymer Science*, *81*, 1588–1594.

Pollock, D. J., & Ktatz, R. F. (1980). R. A. Fava (Ed.), *Methods of experimental physics*. London, United Kingdom: Academic Press Inc.

Suganuma, T., Kitazono, J. I., Yoshinaga, K., Fujimoto, S., & Nagahama, T. (1991). Determination of kinetic parameters for maltotriose and higher malto

oligosaccharides in the reactions catalyzed by α-D-glucan phosphorylase from potato. *Carbohydrate Research*, *217*, 213–220.

van der Vlist, J., Palomo Reixach, M., van der Maarel, M. J. E. C., Dijkhuizen, L., Schouten, A. J., & Loos, K. (2008). Synthesis of branched polyglucans by the tandem action of potato phosphorylase and deinococcus geothermalis glycogen branching enzyme. *Macromolecular Rapid Communications*, *29*, 1293–1297.

van der Vlist J. (2011). *Polymerization of hyperbranched polysaccharides by combined biocatalysis*. PhD thesis. The Netherlands: University of Groningen. http://dissertations.ub.rug.nl/FILES/faculties/science/2011/j.van.der.vlist/00titcon.pdf.

Viliaplana, F., & Gilbert, R. G. (2010). Characterization of branched polysaccharides using multiple-detection size separation techniques. *Journal of Separation Science*, *33*, 3537–3554.

Yanase, M., Takaha, T., & Kuriki, T. (2006). α-Glucan phosphorylase and its use in carbohydrate engineering. *Journal of the Science of Food and Agriculture*, *86*, 1631–1635.

Zea, C. J., & Pohl, N. L. (2004). Kinetic and substrate binding analysis of phosphorylase b via electrospray ionization mass spectrometry: A model for chemical proteomics of sugar phosphorylases. *Analytical Biochemistry*, *327*, 107–113.