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Synthesis of highly branched α-glucans with different structures using GH13 and GH57 glycogen branching enzymes

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A B S T R A C T

Glycogen branching enzymes (GBEs) convert starch into branched α-glucan polymers. To explore if the amylose content of substrates effects the structure of the branched α-glucans, mixtures of amylose and amylpectin were converted by four thermophilic GBEs. The degree of branching and molecular weight of the products increased with an increasing percentage of amylose with the GH57 GBEs of Thermus thermophilus and Thermococcus kodakarenisis, and the GH13 GBEs of Rhodothermus marinus and Petrotoga mobilis. The only exception is that the degree of branching of the Petrotoga mobilis GBE products is not influenced by the amylose content. A second difference is the relatively high hydrolytic activity of two GH57 GBEs, while the two GH13 GBEs have almost no hydrolytic activity. Moreover, the two GH13 GBEs synthesize branched α-glucans with a narrow molecular weight distribution, while the two GH57 GBEs products consist of two or three molecular weight fractions.

1. Introduction

Glycogen is produced by many microorganisms as a carbon and energy reserve (Roach, 2002). It is a branched polymer of anhydroglucose residues linked via α-1,4-glycosidic linkages and α-1→4,6-glycosidic bond branches. The branches are synthesized by glycogen branching enzymes (GBEs, EC 2.4.1.18), which cleave an α-1,4-glycosidic linkage in a linear chain and attach the cleaved off fragment onto the 6-hydroxyl group of an anhydroglucose moiety located in a chain segment of α-1,4-linked anhydroglucose residues (Devillers, Piper, Ballicora, & Preis, 2003; Kajitura et al., 2011; H. Takata et al., 2010). GBEs are classified in two glycoside hydrolase (GH) families, 13 and 57 (Blesak & Janecek, 2012; Cantarel et al., 2009; Lombard, Ramulu, Drula, Coutinho, & Henrissat, 2014; Zona, Chang-Pi-Hin, O’Donohue, & Janecek, 2004). Although the GH13 and GH57 GBEs have a different three-dimensional structural fold (Feng et al., 2016; Hayashi et al., 2017; Na, Park, Jo, Cha, & Ha, 2017; Pal et al., 2010; Palomo et al., 2011; Santos et al., 2011), they use the same catalytic steps, involving a double displacement mechanism and a covalent glucosyl-envelope intermediate (Chiba, 1997; Koshland, 1953).

While in-vivo GBEs act on growing α-glucan chains, in-vitro GBEs can be utilized to modify starch, amylose, and amylopectin. GBE modified starch, or (highly) branched maltodextrin, is characterized by the absence of long linear α-1,4-glucan chains and an increased percentage of α-1→4,6 branches. The resulting product is highly soluble in water, and has no tendency to retrograde because of the absence of amylose and long linear α-1,4-linked chains. Various applications for highly branched maltodextrins have been reported, among which paper coating, slowly digestible starch, sport drinks ingredient, spray drying aid, bio-friendly adhesive (Backer & Saniez, 2005; Ellis et al., 1998; Fuertes, Roturier, & Petitjean, 2005; M. J. E. C. Van der Maarel, Ter Veer, Vrieling-Smit, & Delnoye, 2014).

Currently, only the Rhodothermus obamensis GBEs is commercially available, under the tradename Branchzyme® from Novozymes. However, many more bacterial GH13 GBEs have been characterized, among which the GBEs of Aquifex aeolicus (Hiroki Takata, Ohdan, Takaha, Kuriki, & Okada, 2003; M. Van Der Maarel, Vos, Sanders, & Dijkhuizen, 2003), Butyrivibrio fibrisolvens (Rumbak, Rawlings, Lindsey, & Woods, 1991), Deinococcus geothermalis and Deinococcus radiodurans (Palomo, Kralj, van der Maarel, & Dijkhuizen, 2003), Geobacillus stearotherophilus (Hiroki Takata et al., 1994), Geobacillus thermoglucosidans (Ban et al., 2016; Liu et al., 2017), Mycobacterium tuberculosis (Garg, Alam, Kishan, & Agrawal, 2007), Thermomonospora curvata (Fan, Xie, Zhan, Chen, & Tian, 2016), and Vibrio vulnificus (Jo, Park, Jeong, Kim, & Park, 2015). Today only three GH57 GBEs have been characterized; Pyrococcus horikoshii (Na et al., 2017), Thermococcus kodakarenisis (Murakami, Kanai, Takata, Kuriki, & Imanaka, 2006), and Thermus thermophilus (Palomo et al., 2011). The biochemical studies
have reported variations in substrate specificity, with the GH57 GBEs having a higher catalytic activity with amylose than amylpectin, a substrate preference not seen with the GH13 GBEs. The GH57 GBEs have, in addition, a significant hydrolytic side activity, whereas the GH13 GBEs show hardly any hydrolytic activity (Jo et al., 2015; Palomo et al., 2009, 2011). The differences in reaction specificity are potentially advantageous in the starch processing industry, as it may lead to different types of highly branched α-glcan products.

Here the synthesizes of branched α-glucan by four thermophilic GBEs is explored, revealing that the structure of the branched α-glucans is dependend on the amylose content of the substrate and the GBE used.

2. Materials and methods

2.1. Materials

Amylose V and waxy potato starch (Eliane 100) were provided by Avebe (Veendam, Netherlands). Isoamylase (EC 3.2.1.68, specific activity 260 U/mg), pullulanase M1 (EC 3.2.1.41, specific activity 34 U/mg) and β-amylose (EC 3.2.1.2, specific activity 10,000 U/mL) were obtained from Megazyme (Wicklow, Ireland). General chemicals were obtained from Sigma Aldrich (Darmstadt, Germany) and VWR (Amsterdam, Netherlands).

2.2. Production and purification of glycogen branching enzymes

Codon optimized genes encoding the GBEs from T. thermophilus HB8 (Tggb57) and T. kodakarensis KOD1 (Tkgb57) were synthesized by Baseclear (Leiden, The Netherlands), cloned into the pRSET A (Thermo Fisher Scientific, Waltham) expression vector and overexpressed in E. coli BL21 (DE3). The codon optimized genes encoding the GBEs from R. marinus (Rmgb13) and P. mobiles (Pmgbe13) were synthesized by GeneScript (Hong Kong, China), and cloned into the pET28a expression vector and overexpressed in E. coli BL21 (DE3). The four encoded proteins carry a 6×His-tag at their N-terminus. E. coli was cultivated in Luria-Bertani (LB) medium (10 g/L of tryptone, 5 g/L yeast extract, and 10 g/L NaCl). 100 μg/mL ampicillin was supplemented for Tggb57 and Tkgb57 expression and 50 μg/mL kanamycin was applied for Rmgbe13 and Pmgbe13 expression. Gene expression was induced by 0.1 mM IPTG at 18 °C, and cultivation was continued for 20 h with shaking at 150 rpm. The cells were harvested by centrifugation (5000×g, 10 min, 4 °C), washed twice with 5 mM phosphate buffer pH 7.0, and resuspended in binding buffer (20 mM sodium phosphate, 500 μM NaCl, and 20 mM imidazole, pH 7.4). Cells were lysed using a high-pressure homogenizer (Emulsionflex-B15; Avetin, Ottawa, Canada). The soluble fraction of the cell lysates was collected by centrifugation (20,000×g, 20 min, 4 °C). The proteins were purified in two steps. The soluble fraction was incubated at 65 °C for 10 min, followed by removal of denatured proteins by centrifugation (20,000×g, 20 min, 4 °C); the heat treatment was repeated once. Subsequently, the His-tagged proteins were purified using the HisPurTM Ni-NTA Resin according to the manufacturer’s protocol. Protein concentrations were quantified using the Quick Start Bradford Protein Assay kit (Bio-Rad Laboratories, Veenendaal, Netherlands). The purity and molecular mass of the proteins were check by SDS-PAGE.

2.3. Enzyme activity assays

Amylose V, dissolved in 1 M NaOH, and then neutralized to pH 7.0 with 1 M HCl, was used as a model substrate. Branched α-glucan products were prepared at the optimal reaction conditions: 0.125% (w/v) substrate, 35 μg/mL Tggb57, 65 °C, pH 6.5; 30 μg/mL Tkgb57, 70 °C, pH 7.0; 3.0 μg/mL Rmgb13, 65 °C, pH 7.0 and 3.0 μg/mL Pmgb13, 50 °C, pH 7.0.

Branching activity, representing the newly synthesized 1,6-glycosidic linkages, was quantified by measuring the increase in reducing ends upon debranching of the product. The amount of reducing ends was measured by bicinchoninic acid (BCA) method (Waffenschmidt & Jaenicke, 1987). Briefly, 0.125% amylose V was incubated, with different GBEs, in 50 mM sodium phosphate buffer, as described above. Samples of 200 μL were taken at regular time intervals and the reaction was stopped by boiling for 10 min. Of the samples taken, 50 μL was debranched by adding 1 μL of 0.1 M HCl (which lowers the pH to 5.0), 0.7 U isoamylase, 0.5 U pullulanase and 5 mM CaCl2, and incubating at 40 °C for 16 h. The hydrolytic activity was also quantified, by following the increase in reducing ends within the reaction mixture in time. Branching activity is simply the difference in the amount of reducing ends after debranching minus the amount of reducing ends before debranching. One unit of branching activity is defined as 1 μmol of branches formed per minute (Palomo et al., 2011). The branching activity of Tggb57, Tkgb57 and Pmgb13 on amylose was calculated from the increase in branches formed within the first 30 min (10 min for Rmgb13), while the hydrolytic activity is calculated from the increase in reducing ends over 24 h. The branching activity on amylopectin was calculated from the increase in branches formed within the first 15 min, while the hydrolytic activity is calculated from the increase in reducing ends over 24 h.

2.4. High performance anion exchange chromatography

Oligosaccharide analyses was carried out by High Performance Anion Exchange Chromatography (HPAEC) on a Dionex ICS-3000 system (Thermo Fisher Scientific) equipped with a 4×250 mm CarboPac PA-1 column. A pulsed amperometric detector with a gold electrode and an Ag/AgCl pH reference electrode was used. The system was run with a gradient of 30–600 mM NaAc in 100 mM NaOH 1 mL/min. Chromatograms were analyzed using Chromelone 6.8 chromatography data system software (Thermo Fisher Scientific, Waltham). A mixture of glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltotetraose, and maltotetraose was used as reference for qualitative determination of elution time of each component.

The chain length distribution was determined by the debranching the branched α-glucans (2 mg/mL in 5 mM sodium acetate buffer pH 5.0) with 0.7 U/mL isoamylase and 0.5 U/mL pullulanase at 40 °C for 16 h. The debranching reactions were stopped by boiling for 5 min, and denatured proteins were removed by centrifugation, and analyzed by HPAEC.

2.5. 1H-NMR spectroscopy

1H-NMR spectra were recorded at a probe temperature of 323 K on a Varian Inova 500 spectrometer (NMR Center, University of Groningen). Before analysis, samples were exchanged twice in D2O (99.9 atom% D, Sigma-Aldrich Chemical) with intermediate lyophilization, and then dissolved in 0.6 mL D2O. Spectra were processed using MestReNova 5.3 software (Mestrelabs Research SL, Santiago de Compostella, Spain).
using Whittaker Smoother baseline correction and zero filling to 32 k complex points. Carbohydrate structures were determined using the previously developed 1H-NMR structural-reporter-group concept of α-D-glucans (van Leeuwen, Lee flang, Gerwig, & Kamerling, 2008). The α-1,6-signal is presented at δ 4.98, originating from H1 in 1,4-α-glucose-1,6, and α-1,4-signal is at δ 5.36 from the H1 in 1,4-α-glucose-1,4 and 1→4,6-α-glucose-1,4 residues. The degree of branching (α-1,6-linkage ratio) was calculated by dividing the area of α-1,6-linkage peak by the total area of α-1,4-linkage and α-1,6-linkage peaks in the NMR spectra.

2.6. GPC-SEC

Molecular weight distributions were measured by GPC-SEC run with DMSO-LiBr. DMSO-LiBr (0.05 M) was prepared by stirring for 3 h at room temperature. Samples were dissolved at a concentration of 2 mg/mL in DMSO-LiBr at 80 °C for 3 h with shaking and then filtered through a 0.45 μm Millex PTFE membrane (Millipore Corporation, Billerica, USA). The Size Exclusion Chromatography (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, Santa Clara), viscometer (ETA-2010 PSS, Mainz, Germany), and MALLS (SLD 7000 PSS, Mainz, Germany). WinGPC Unity software (PSS) was used for data processing. The samples were injected with a flow rate of 0.5 mL/min into a PFG guard-column and three PFG SEC columns 100, 300 and 4000 (PSS). The columns were held at 80 °C, and the detectors were held at 60 °C (Visco) and 45 °C (RI). A standard pullulan kit (PSS) 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 was measured by PSS and is 0.072.

2.7. Fractionation of branched α-glucans

The branched α-glucans obtained from amylose by TtGBE57 modification were separated into a high and low molecular weight fractions using size-exclusion chromatography (Hiprep 26/60 Sephacryl S-500 h column). Briefly, 25 mg branched α-glucan in 5 mL was injected, and the column was run at 2.5 mL/min water with 0.02% NaN3. The collected fractions were freeze dried, and subsequently analyzed by NMR and GPC-SEC, as described above.

2.8. Modification of different amylose content substrates

The mixtures of amylose and amylpectin were prepared by combining solutions of amylose V and waxy potato starch at different ratios. Firstly, 5 mg/mL amylose V was dissolved into 1 M NaOH, and then neutralized to pH 7.0. Waxy potato starch (7.5 mg/mL) was dissolved in 50 mM phosphate buffer with pH 7 by boiling. The enzymatic reaction conditions were as above. The reactions were stopped by boiling for 10 min, the denatured proteins removed by centrifugation, and the supernatant dialyzed using a dialysis tube with a cutoff size of 100–500 Da. The samples were freeze dry and analyzed, as described

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**Table 1**
The branching and hydrolytic activities of GBEs.

<table>
<thead>
<tr>
<th>GBEs</th>
<th>Amylose as substrate</th>
<th>Amylopectin as substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Branching activity (mU/mg)</td>
<td>Hydrolytic activity (mU/mg)</td>
</tr>
<tr>
<td>TtGBE57</td>
<td>490</td>
<td>1.7</td>
</tr>
<tr>
<td>TkGBE57</td>
<td>560</td>
<td>2.6</td>
</tr>
<tr>
<td>PmGBE13</td>
<td>6,100</td>
<td>7.5</td>
</tr>
<tr>
<td>RmGBE13</td>
<td>18,700</td>
<td>7.1</td>
</tr>
</tbody>
</table>

---

**Fig. 2.** Branching and hydrolysis reaction progress. Reaction progress is followed by quantifying the increase in reducing ends over time in the conversion of amylose V. TtGBE57 (A); TkGBE57 (B); RmGBE13 (C) and PmGBE13 (D). Black dot: the reducing end concentration increase upon debranching.
3. Results and discussion

3.1. Branching and hydrolytic activity of glycogen branching enzymes

Two GH57 gbe genes, from *T. thermophilus* and *T. kodakarensis*, and two GH13 gbe genes, from *R. marinus* and *P. mobilis*, were overexpressed in *E. coli* and the corresponding GBEs were purified by a combination of heat treatment and His-tag affinity chromatography (Fig. 1). The two GH13 GBEs have a considerable higher branching activity than the two GH57 GBEs, using amylose V and amylopectin as substrates (Table 1). The two GH57 GBEs have a slightly higher branching activity on amylose, the RmGBE13 shows 40% more branching activity with amylopectin, and PmGBE13 has two times more branching activity on amylose.

In addition to the branching activity, GBEs also possess a low hydrolytic activity, which results in the formation of oligo and polysaccharides smaller than the substrate. Acting on amylose V, both GH57 GBEs have a considerably lower ratio of branching over hydrolytic activity than the two GH13 GBEs (Table 1), which is in agreement with previous publications (Palomo et al., 2009, 2011). Indeed, upon prolonged incubation of amylose V with the two GH57 GBEs, about 15% of the catalytic actions had resulted in the formation of a new reducing end (hydrolysis activity), and 85% in the formation of a new branch (branching activity) (Fig. 2). The two GH57 GBEs are even more hydrolytic with amylopectin as substrate, with 40% hydrolysis and 60% branching (Supplementary Fig. S1). The two GH13 GBEs, in contrast, formed almost only branches, with both amylose and amylopectin (Fig. 2 and Supplementary Fig. S1). Indeed, HPAEC analysis shows substantial amounts of short oligosaccharides in the products made by the two GH57 GBEs (Fig. 3), which are basically absent in the products made by the two GH13 GBEs. Thus, the two GH57 GBEs are far more hydrolytic than two GH13 GBEs, when acting *in-vitro* on pre-existing α-glucan polymers. Although out of scope for this publication, it remains an intriguing question whether GH57 GBEs are also relative hydrolytic *in-vivo*.

3.2. The structure of branched α-glucan made by GBEs is influenced by the amylose to amylopectin ratio

As native starches are composed of amylpectin and amylose, with the amylose content ranging from as low as 0 up to 70% in commercially available starches (Jane et al., 1999), it was explored if the amylopectin to amylose ratio affects the structure of the branched α-glucan synthesized. Therefore various ratios of amylose and amylpectin were incubated with the four GBEs and the obtained products were analyzed in detail. Firstly, the chain length distribution of the branched α-glucans derived from 100% amylose and 100% amylopectin were compared. This revealed that the two GH57 GBEs form products with a wider chain length distribution. While the products of the two GH13 GBEs explored here have very similar chain length distribution, not all GH13 GBEs synthesize similar products. For example, the potato SBEI and SBEII GH13 branching enzymes form branched α-glucans with distinct chain length distributions (Rydberg, Andersson, Andersson, Aman, & Larsson, 2009).
Subsequently the degree of branching of all samples was determined, demonstrating that the two GH13 GBEs form products with a higher degree of branching. Surprisingly, it was also observed that the degree of branching is positively correlated with the percentage of amylose in the substrate with the two GH57 GBEs (Fig. 5). The same correlation is seen with the RmGBE13, although the effect is somewhat milder, while the effect is absent with the PmGBE13 (Fig. 5). Since the initial branching activity of the two GH57 GBEs with amylose and amylopectin are quite similar (Table 1), the much higher degree of branching reached with amylose indicates that the branches are distributed more efficiently starting with amylose, compared to amylopectin. However, a previous study reported that GBE formed slightly less branched α-glucans with increasing amylose content of the substrate (Sorndech et al., 2016). Sorndech et al. used high Mw (5500 kDa) barley amylose and waxy maize amylopectin, while in the current study potato amylose with a much lower Mw (100 kDa) was used. Possibly branching enzymes are more effective with shorter substrates.

### 3.3. Branched α-glucans derived from amylose and amylopectin have different molecular weight profiles

Besides the degree of branching and the chain length distributions also the molecular weight (Mw) is a key structural parameter of branched α-glucans. To determine if the Mw is influenced by the amylose content of the substrate the branched α-glucans derived from amylose and amylopectin were analyzed by GPC. The first observation is that the four GBEs synthesize branched α-glucans with distinct Mw distributions. Acting on amylose the two GH13 GBEs generate products with a relative mono disperse Mw distribution (Fig. 6); \( \sim 1 \times 10^5 \) Da for RmGBE13 and \( \sim 2 \times 10^5 \) Da for PmGBE13. The two GH57 GBEs surprisingly generated products consisting of a low and a high Mw fraction (Fig. 6). Separation of the high and low Mw fractions of the TtGBE57 product, using size-exclusion column chromatography, revealed that the low Mw fraction (\(< 4 \times 10^4 \) Da) contains only 5% branches and the high Mw fraction (\( > 7 \times 10^4 \) Da) contains 10% branches. This indicates that the low Mw fraction consist mainly of the products resulting from hydrolysis with mild branching, whereas the high Mw fraction is the result of the branching reaction.

The picture is distinctively different with amylopectin as substrate. The most remarkable difference being the lower Mw of the branched α-glucans derived from amylopectin compared to the products derived
from amylose. Subsequently the mixtures of amylose and amylopectin were converted by the four GBEs revealing that the average $M_w$ increases with increasing amylose content. The same phenomena has also been observed for the GH13 GBE from *Rhodothermus obamensis* (Sorndech et al., 2016), and now also in this study. From these observations it is concluded that GBEs convert the high $M_w$ amylopectin into branched $\alpha$-glucans with lower $M_w$, whereas amylose is converted into branched $\alpha$-glucans with a $M_w$ corresponding to that of amylose. The $M_w$ distributions of the branched $\alpha$-glucans derived from amylose and amylopectin show, however, also similarities. The GH13 RmGBE13 product is again rather mono disperse, whereas the two GH57 products consist of two or three $M_w$ fractions (Fig. 6). The GH13 PmGBE13 derived from amylopectin product is special in the sense that consist of two $M_w$ fractions, while the enzyme converts amylose in a mono disperse branched $\alpha$-glucan (Fig. 6). Thus, the different GBEs create highly branched $\alpha$-glucan with various $M_w$ distributions, and the GH13 RmGBE13 is the best choice for the production of product with a uniform $M_w$ distribution.

3.4. Synthesis of branched $\alpha$-glucans from a waxy starch and a high amylose starch

The question arises if the trends seen with the artificial mixtures of amylose and amylopectin also hold in the conversion of natural starches, which vary widely in their amylose content, (Knutson & Grove, 1994; Morrison & Laignelet, 1983). To test this waxy corn and a high amylose pea starch (with 35% amylose) (Jensen et al., 2013; Lourdin, Della Valle, & Colonna, 1995) were treated with the GBEs. NMR analysis of the branched $\alpha$-glucans obtained demonstrated that the degree of branching of these products (Table 2) is in good agreement with the values obtained with the artificial mixtures of amylose or amylopectin (Fig. 5). Thus, the two GH13 GBEs synthesized branched $\alpha$-glucans with a higher degree of branching than the two GH57 GBEs (Table 2). And secondly, the two GH57 GBEs create products with a higher degree of branching from the amylose rich pea starch than from the waxy maize starch, as predicted by the model system employing a mixture of amylose and amylopectin.

4. Conclusions

Here we report the synthesis of branched $\alpha$-glucans by four thermophilic GBEs. Whereas amylose is converted in highly branched $\alpha$-glucans by all four GBEs, conversion of amylopectin yields highly branched and modest branched products with the GH13 and GH57 GBEs, respectively. The two GH13 GBEs products have a narrow

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**Table 2**
The DB of pea and waxy corn starches following modifications with *T. thermophilus, T. kodakarensis, R. marinus,* and *P. mobilis* GBEs. Based on the results of triplicate analyses.

<table>
<thead>
<tr>
<th>GH Family</th>
<th>GBE</th>
<th>Pea starch</th>
<th>Waxy corn starch</th>
</tr>
</thead>
<tbody>
<tr>
<td>57</td>
<td>TtGBE57</td>
<td>6.2 ± 0.44</td>
<td>5.3 ± 0.65</td>
</tr>
<tr>
<td></td>
<td>TkGBE57</td>
<td>6.2 ± 0.64</td>
<td>5.0 ± 0.21</td>
</tr>
<tr>
<td>13</td>
<td>RmGBE13</td>
<td>10.9 ± 0.20</td>
<td>10.2 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>PmGBE13</td>
<td>13.1 ± 0.30</td>
<td>12.7 ± 0.49</td>
</tr>
</tbody>
</table>

Fig. 6. The molecular weight distribution of the branched $\alpha$-glucans derived from amylose and amylopectin mixtures by TtGBE57 (A), TkGBE57 (B), RmGBE13 (C) and PmGBE13 (D) treatment. The analysis was performed by GPC-MALLS-RI run with DMSO containing 50 mM LiBr.
molecular weight distribution, while the two GH57 GBEs products have a wide molecular weight distribution. We speculate that the production of branched α-glucans with a narrow molecular weight distribution requires GBEs with very low hydrolytic activity. Indeed, the commercially available RmGBE13 (Branchzyme, Novozymes) has the highest branching over hydrolysis specificity and synthesizes the branched α-glucan with the most mono disperse molecular weight distribution of the GBEs evaluated.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:10.1016/j.carbolip.2019.04.038.

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