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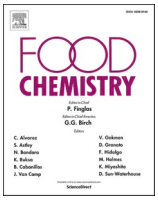

The influence of amylose content on the modification of starches by glycogen branching enzymes

Aline L.O. Gaenssle, Marc J.E.C. van der Maarel, Edita Jurak *

Bioproduct Engineering, Engineering and Technology Institute Groningen, University of Groningen, Nijenborgh 4, 9747 AG, Groningen, The Netherlands

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ABSTRACT

Glycogen branching enzymes (GBEs) have been used to generate new branches in starches for producing slowly digestible starches. The aim of this study was to expand the knowledge about the mode of action of these enzymes by identifying structural aspects of starchy substrates affecting the products generated by different GBEs. The structures obtained from incubating five GBEs (three from glycoside hydrolase family (GH) 13 and two from GH57) on five different substrates exhibited minor but statistically significant correlations between the amount of longer chains (degree of polymerization (DP) 9–24) of the product and both the amylose content and the degree of branching of the substrate (Pearson correlation coefficient of ≤ -0.773 and ≥ 0.786, respectively). GH57 GBEs mainly generated large products with long branches (100–700 kDa and DP 11–16) whereas GH13 GBEs produced smaller products with shorter branches (6–150 kDa and DP 3–10).

1. Introduction

Glycogen branching enzymes (GBEs, EC 2.1.418) are widely distributed proteins active on glycogen (Abad et al., 2002) and are present in two glycoside hydrolase (GH) families, the family GH13 and GH57 (Janeček & Gabriško, 2016; Suzuki & Suzuki, 2016). These enzymes have been described to catalyze the formation of branches by first cleaving an α-1,4 glycosidic bond within a linear chain segment followed by the attachment of the cleaved fragment onto another linear chain by formation of an α-1,6-glycosidic linkage (Kuriki & Imanaka, 1999). GBEs from the two GH families share a structurally similar catalytic domain ($(\alpha/b)_8$-barrel and $(\alpha/\beta)_7$-barrel for GH13 and GH57, respectively) but differ in their other two domains (domain N and C) (Abad et al., 2002; Pal et al., 2010; Palomo et al., 2011). Furthermore, one of the three catalytic residues of GH13 GBEs is missing in GH57 GBEs (Janeček & Gabriško, 2016; Palomo et al., 2011) although their catalytic mechanisms are assumed to be similar (Palomo et al., 2011).

For application, enzymatic, chemical and physical methods have been shown to effectively alter starch structure. GBEs are e.g. used in industry to generate slowly digestible starches (Sorndech et al., 2018) hence the most typically used substrate for GBEs is amylose (Jo et al., 2015; Zhang et al., 2019a) followed by *ae*-amylopectin from rice (Sawada et al., 2014). Next to enzymatic modification, recently, physical methods with ultrasonic and electric field modifications were shown to modify the amorphous and crystalline regions of potato starch (Cao & Gao, 2020). Chemical modifications, such as acid hydrolysis and succinylation were also demonstrated to affect crystallinity, viscosity and thermal properties of corn starch (Basilio-Cortés et al., 2019). Starch is entirely built up of glucose residues linked by α-1,4- and α-1,6-glycosidic linkages where the first bond type forms linear chains and the second branch points (Tetlow & Emes, 2014). It consists of two components, the smaller, mostly linear amylose and the larger, branched amylopectin with an amylose content typically ranging from 15 to 30% (Waterschoot et al., 2015). Starches from distinct plant origins differ from each other in various structural properties such as crystalline type (Sarko & Wu, 1978), amylose content (Waterschoot et al., 2015), degree of branching (Nilsson et al., 1996) and average chain length (Vamadevan & Bertoft, 2015).

GBEs have been studied for many years with main focus on the characterization of optimal conditions of novel enzymes working on

Abbreviations: BfGBE, GBE from *Butyrivibrio fibrisolvens*; DP, degree of polymerization; GBE, glycogen branching enzyme; GH13, glycoside hydrolase family 13; GH57, glycoside hydrolase family 57; kDa, kilo Daltons (1 kDa = 1 kg/mol); MD-Mix, maltodextrin mixture of DP 10–40; PmGBE1, GBE1 from *Petrotoga mobilis*; RmGBE, GBE from *Rhodothermus marinus*; S_AF/LF ratio, ratio between the accumulated peak areas of the chain fractions DP 9–12 (short fraction, S_AF) and DP $\geq$ 13 (long fraction, LF); TkGBE, GBE from *Thermococcus kodakarensis*; TtGBE, GBE from *Thermus thermophilus*.

* Corresponding author.
E-mail addresses: a.l.o.gaenssle@rug.nl (A.L.O. Gaenssle), m.j.e.c.van.der.maarel@rug.nl (M.J.E.C. van der Maarel), e.jurak@rug.nl (E. Jurak).

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starches and the properties of their products (Jo et al., 2015; Murakami et al., 2006; Yoon et al., 2008; Zhang et al., 2019a). Therefore, most of the studies were reported for a single GBE and substrate (typically amylose) (Takata et al., 2003; Yoon et al., 2008). Only a few studies are available to date that report an in-depth characterization of either several GBEs from distinct organisms (Palomo et al., 2009; Sawada et al., 2014; Zhang et al., 2019b) or one GBE on starches of different plant origins (Li et al., 2018) but none that did both. These studies have shown that GBEs exhibit substantial variations both between each other and on different substrates such as a preferences for either amylose or amylopectin (Jo et al., 2015; Zhang et al., 2019b). Limiting the study to one enzyme or substrate makes it difficult to extrapolate the observed behavior to identify the key components effecting the mode of action of GBEs. Understanding of GBEs activity pattern, however, is crucial in predicting the obtained products as required in industry. This is especially important as both the substrate and product of GBEs are highly complex structures hampering the interpretation of the results. The hypothesis of this study was that specific structural features of the starch substrates, such as amylose content and/or degree of branching will influence the structure of products obtained from GBE modification. The aim is to investigate whether glycogen branching enzymes from different families and previously proposed different catalytic mechanism would produce different product from each other and also whether starch structural differences play a part in the extent of modification for these enzymes.

For this purpose, three GH13 GBEs, originating from *Butyrivibrio fibrisolvens* (BfGBE), *Petrotoga mobilis* (PmGBE1), *Rhodothermus marinus* (RmGBE). These GBEs were selected as they showed substantial differences in their catalytic behavior as well as the obtained chain lengths of their products (Gaenssle, Bax, & van der Maarel, 2021). In addition to the three GH13 GBEs, two GBEs, being from *Thermococcus kodakarensis* (TkGBE) and *Thermus thermophilus* (TtGBE), were selected for characterization as GBEs from the two families have been described to generate distinct products (Zhang et al., 2019b). All five GBEs have been characterized previously regarding their optimal conditions and activity on potato amylose (Murakami et al., 2006; Palomo et al., 2011; Rumbak et al., 1991; Yoon et al., 2008; Zhang et al., 2019a). Further, five substrates were selected which were four starches (potato, tulip, wrinkled pea and waxy corn starch) as well as a mixture of linear chains (degree of polymerization 10–40). These starches had been analyzed previously and were selected based on their fundamental differences in structure, such as crystalline type, amylose content (21.9–63.6%), degree of branching (0.4–6.0%) and ratio between short (DP $<$ 13) and long (DP $\geq$ 13) chains (Gaenssle, Satyawan, Xiang, van der Maarel, & Jurak, 2021).

2. Material and methods

2.1. Material

Potato starch (food grade) was obtained from AVEBE (the Netherlands), waxy corn starch (Meritena® 300) from Tereos Syral (France) and Maltodextrin High DP (GLU310) from Elicityl-Oligotech (France). Tulip bulbs and wrinkled pea seeds were purchased at local shops and the starches were extracted as described previously (Gaenssle, Satyawan et al., 2021). The amylose content of the used starches had been determined previously with the amylose/amylopectin assay kit purchased from Megazyme (Ireland) (Gaenssle, Satyawan et al., 2021).

Isoamylase from *Pseudomonas* sp. (E-ISAMY, 200 U/ml) and pullulanase M1 from *Klebsiella planticola* (E-PULKP, 650 U/ml) were both obtained from Megazyme (Ireland). MagicMedia and HisPur™ Ni-NTA Resin were purchased from ThermoFischer Scientific (USA). A pullulan kit (0.34–708 kDa, 1 kDa = 1 kg/mol) was obtained from PSS. All chemicals were of analytical grade or higher.

2.2. Production and purification of enzymes

The glycogen branching enzymes (GBEs) BfGBE and PmGBE1 were produces and purified as describes previously (Gaenssle, Bax, & van der Maarel, 2021). TkGBE and TtGBE were kindly gifted by Xian et al. (2022). After purification, the enzyme samples underwent a buffer exchange using PD-10 Desalting columns (GE Healthcare) at RT. First, the column was equilibrated with 25 ml HEPES buffer (20 mM, pH 7.4), and centrifuged for 2 min at 1000×*g* before adding 2.5 ml enzyme solution followed by centrifugation for 2 min at 1000×*g*.

2.3. Basic enzyme characterization

The protein content was determined by Bradford, with bovine serum albumin (BSA) as standard and the purity with sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) stained with Coomassie Brilliant Blue.

The activity was determined with the iodine assay (Gaenssle et al., 2020). The 200 µl enzyme reactions contained 2–20 µg/ml GBE, 1 mg/ml amylose (from 100 mg/ml in 100% dimethyl sulphoxide stock solution) in 50 mM sodium phosphate buffer, pH 6.0. Every full minute, 15 µl aliquots from the enzyme reaction were mixed with 100 µl iodine reagent (0.26% KI, 0.026% I_2, 5 mM HCl). The absorbance was detected at 610 nm using a spectrophotometer (SpectraMax from Molecular Devices).

2.4. Enzyme reactions

Five different substrates (waxy corn, potato, tulip, wrinkled pea starch and maltodextrin mixture) were solubilized to 6.2 mg/ml in 15 mM phosphate buffer, pH 7.2 by incubating them for 30 min in a 95 °C water bath with frequent mixing. The branching reaction was conducted by incubating the five GBEs with all five substrates in triplicates, negative controls for substrate and enzyme were included as well. The reactions were carried out using 25 µg/ml enzyme and 5 mg/ml substrate in 450 µl 12 mM sodium phosphate buffer, pH 7.2. All samples were incubated slowly rotating for 24 h with the samples of BfGBE being incubated RT and all others at 50 °C.

For the debranching reaction, the samples were diluted twice in sodium citrate buffer, pH 4.5 and incubated with isoamylase and pullulanase (each 1 U/mg substrate) for 24 h at 40 °C, slowly rotating. For the starches, the debranching was repeated twice with replenished the debranching enzymes.

2.5. Size exclusion chromatography

Products derived from GBE modification were centrifuged (10,000×*g*, 10 min) and the supernatant was diluted to 2 mg/ml α-glucan. The *SEC* system (Agilent Technologies 1200 Series) was equipped with three Suprema PSS columns (100 Å, 1000 Å and 3000 Å; 300 × 8 mm 10 µm) tempered at 40 °C and a refractive index detector. Samples (injection volume 10 µl) were eluted with a flow rate of 1 ml/min using 0.05 M $NaNO_3$ as eluent. Ethylene glycol was used as internal standard and a series of pullulan standards (1–708 kDa, 9 standards) as universal standard. The buffer was subtracted from the samples. Samples were analyzed in duplicates and a representative chromatogram was selected for each.

2.6. Anion exchange chromatography

Enzymatically debranched samples (see above) were centrifuged (10,000×*g*, 10 min) and the supernatant was diluted to 1 mg/ml for the starches and 0.5 mg/ml for the maltodextrin mixture. The samples were analyzed with High-Performance Anion Exchange Chromatography coupled with Pulsed Amperometric Detection (HPAEC-PAD) using a Dionex ICS-6000 system (ThermoFischer Scientific) with a CarboPac™

PA1 column. The injection volume was 10 µl and the flow rate was 0.3 ml/min. The samples were eluted using the eluents A (0.1 M NaOH) and B (0.1 M NaOH, 1 M NaOAc) with the following gradient profile: 0–50 min (5–40% B), 50–65 min (40–100% B), 65–70 min (100 % B), followed by a re-requilibration at 5% B. The peak areas were calculated using the Chromelion software version 7.2.9 and samples were represented by mean (n = 2). Identification of peaks was performed by using maltodextrin mixture of known molecular weights,

2.7. Computational analysis

The sequence alignment was conducted using the BlastP software from NCBI (Altschul et al., 1990). The statistical analysis was conducted in Stata16 (StataCorp. USA) using the Pearson correlation procedure with a confidence interval of 99.5%.

3. Results and discussion

Five different branching enzymes have been selected, originating from *B. fibrisolvens* (BfGBE), *P. mobilis* (PmGBE1), *R. marinus* (RmGBE) from family GH13 and *T. kodakarensis* (TkGBE) and *T. thermophiles* (TtGBE) from family GH57. The three GH13 enzymes share around 50% sequence identity (52.5% between PmGBE1 and RmGBE, 50.3% BfGBE and PmGBE1, and 46.7% BfGBE and RmGBE) and the two GH57 enzymes exhibit 44.6% sequence identity. All enzymes were thermostable with exception of BfGBE which was most active at ambient temperatures (Murakami et al., 2006; Palomo et al., 2011; Rumbak et al., 1991; Yoon et al., 2008; Zhang et al., 2019a).

Previously (Gaenssle, Satyawan et al., 2021), we have characterized a number of starchy substrates regarding properties such as crystal type, amylose content, degree of branching, chain length distribution and digestion rate. Based on the thereby obtained data, four starches were selected which exhibited the most pronounced differences from each

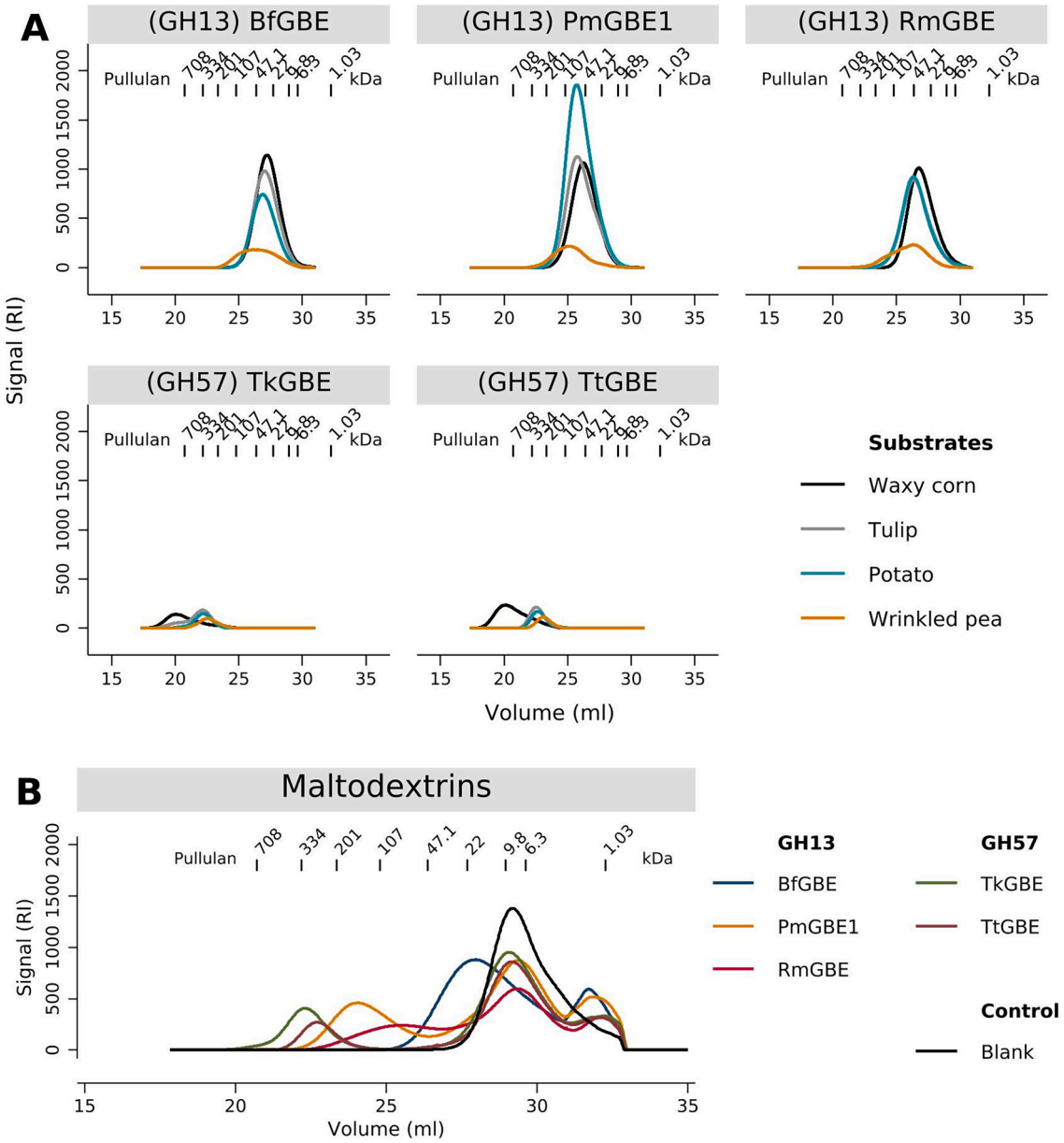

Fig. 1. Size exclusion profiles of products obtained from incubation of five branching enzymes for 24 h with (A) four starches and (B) a mixture of linear maltodextrins (DP 10–40). The reaction and analysis were conducted in triplicates of which one representative replicate is displayed here. Substrates in Figure (A) are sorted from low to high amylose content as well as high to low degree of branching.

other. Waxy corn was selected as A-type crystalline starch, both tulip and potato as B-type starches and wrinkled pea as a representative of the C-type starches (mixture of A- and B-type). Wrinkled pea had by far the highest amylose content (wrinkled pea (63.6%) ≫ tulip (22.7%) > potato (19.5%) ≫ waxy corn (1.9%)) as previously determined with the amylose/amylopectin assay kit purchased from Megazyme. Further, wrinkled pea starch had the lowest degree of branching (wrinkled pea (0.4%) ≪ potato (2.6%) < tulip (3.2%) ≪ waxy corn (6.0%)) and a moderate relative number of short chains (DP < 13; potato > wrinkled pea > waxy corn ≫ tulip) (Gaenssle, Satyawan et al., 2021). This selection of substrates assured that the obtained data could be extrapolated towards other starches.

3.1. Molecular weight distribution of GBE products

As seen in Fig. 1A, all treatments of starch with a GBE resulted in the formation of a single population of product. GH13 GBEs generated populations of considerably smaller size (about 6–150 kDa based on pullulan standard) compared to GH57 GBEs (about 100–700 kDa (1 kDa = 1 g/mol) and larger).

Strikingly, all GBEs generated highly similar populations regardless of the starch used as substrate. For GH13 GBEs, treatment of wrinkled pea generally gave rise to the largest population, followed by modification of potato, tulip and waxy corn, respectively. This pattern coincided with the degree of branching of the substrates (Gaenssle, Satyawan et al., 2021), thus a higher degree of branching resulted in a population of smaller size. Similarly, it further showed a negative correlation between the amylose content of the substrate and the population size of the product. Overall, the peak of the populations shifted from about 40 kDa with wrinkled pea to about 28 kDa for waxy corn. The approximate size of the products of GH13 GBEs was in agreement with the size obtained from RmGBE and PmGBE (Zhang et al., 2019b).

Surprisingly, the products of GH57 enzymes followed the opposite trend, showing a positive correlation between product size and degree of branching (waxy corn ≫ tulip > potato > wrinkled pea) as well as a negative correlation to the amylose content of the substrate. The products obtained with waxy corn were so large that its size could not be estimated with the pullulan standard (Fig. 1A). A previous study on a variant of TkGBE with a deleted C-terminal on amylose AS-30 resulted in two populations, one at 3000–3500 kDa and the other at 4.8–20 kDa (Murakami et al., 2006). Similarly, incubation of TkGBE and TtGBE with a mixture of potato amylose and amylopectin resulted in the formation of three populations (Zhang et al., 2019b). The difference in populations could be due to the substrate (pure amylose and amylopectin versus native starches), the incubation conditions and enzyme dosage. In the here presented study, all enzymes were incubated with the same enzyme dosage (mg protein/dry matter of substrate) and conditions with exception of samples with BfGBE being performed at RT. This was conducted to prevent data distortion as GBEs have been previously described to vary in activity towards different substrates (Zhang et al., 2019b).

The substantial variation in behavior of GBEs from the two families can likely be attributed to the differences in structure of the enzymes such as their catalytic site and overall composition (Abad et al., 2002; Janeček & Gabriško, 2016; Pal et al., 2010; Palomo et al., 2011).

In addition to the overall population size of the products obtained from GBE treatment, the amount of product were also influenced by the amylose content of the substrate (Fig. 1A), exhibiting a clear decrease in product in response to a higher amylose content. This could at least partly be due to the relative insolubility of amylose in aqueous solutions (Green et al., 1975), resulting in possibly reduced enzyme activity due the inaccessibility of the substrate or due to only partial analysis of the products. Small amounts of aggregates were found in all samples which were removed by centrifugation prior analysis, indicating that a minor fraction of the product could not be analyzed. The product obtained from PmGBE1 on potato starch did not follow this trend, giving far larger quantities than for the other samples, which might be due to a very high specificity of PmBE1 for potato starch. GH57 GBEs seemed to be less affected by the high amylose content of wrinkled pea starch as the amount of products obtained from this starch was not substantially less than from the other starches as was the case for GH13 GBEs. Although both the amylose content and degree of branching of the substrates were found to effect the size and amount of the populations of the products, their impact was minor compared to the differences observed between the GBEs from the two enzyme families.

Overall, all GBEs generated products of smaller size than the untreated starches. Possibly, GBEs not only cleave the α-1,4 bonds of external branches but also of long chains connecting the clusters in the amylopectin fine structure, thereby cleaving the large molecules into smaller fractions as has been suggested for e.g. the GH13 GBE from *E. coli* (Sawada et al., 2014). Another possibility is that GBEs transfer chains to loose chains, thereby giving rise to their own structure while the majority of the substrate remains undetected. This possibility was further investigated by incubation with a maltodextrin mixture (MD-Mix, DP 10–40). As seen in Fig. 1B, the untreated mixture showed a single population in the range of 1–14 kDa (pullulan standard) which was broader than expected as the size was equivalent to about DP 6–90. This suggested that the linear chains of the substrates were either larger than indicated or connected to each other by branches.

Interestingly, in contrast to the products obtained from the starches, the modified maltodextrins were distributed into three populations for all but those from BfGBE which resulted in only two populations. The GH13 GBEs BfGBE and RmGBE were most efficient in converting the substrate although all GBEs were capable of modifying the substrate, generating one smaller and one larger fraction. The largest population of each product followed the same pattern as the starches with TkGBE generating the largest product, followed by TkGBE, PmGBE1, RmGBE and BfGBE, respectively. MD-Mix modified with the two GH57 GBEs contained products of about 100–700 kDa, being comparable with the sizes obtained from the treated starches. In contrast, the products obtained from GH13 GBEs on MD-Mix showed different sizes compared to their products from starches. Both PmGBE1 and RmGBE generated larger products (about 25–450 kDa) whereas BfGBE produced populations of smaller size (about 3–70 kDa). For the GH13 GBEs, a positive correlation between the size of the products and the amylose content of the starch substrate was found (Fig. 1A), possibly indicating their preference for linear chains since amylose consists almost entirely of linear chains. Therefore, the observed behavior of PmGBE1 and RmGBE on MD-Mix was in line with their activity on the starches as the mixture of mostly linear chains could be described as pure amylose. BfGBE was the only enzyme that appeared to be capable of modifying the entire population of the substrate. It might further be possible that BfGBE slowly increased its product size over the course of the reaction and would continue to do so if the reaction had been allowed to continue.

Apart from the larger population, all GBEs generated some small oligomers which were further investigated by analyzing the chain length distribution of the branched products (Fig. 4). Taken together, all studied GBEs were capable of using the provided linear chains and generate larger molecules, likely through repeated attachment of branches to the same molecule. Furthermore, the populations obtained from MD-Mix were comparable in size to the products obtained after GBE treatment of the starches. Therefore, it appeared that the structure of the substrate was not essential in defining the size of the product.

3.2. Chain length distribution of modified starches

The analysis of the overall populations was followed by the study of the distributions of the debranched chains from the respective products. The debranching proved to be a challenge as even after a third debranching step branched products were still visible in the chain length profiles (Fig. S1) for all GBE treated samples. Two debranching enzymes were used, isoamylase and pullulanase. Isoamylase appears to be

incapable of cleaving branches shorter than polymerization (DP) 3 while pullulanase has been reported to cleave of branches of even DP 1 but was unable to penetrate clusters of branches (Yokobayashi et al., 1970). Therefore, remaining branched products could indicate very short branches in too close proximity. GBEs have not been described so far of generating such short branches but it is plausible that they might cleave the chains of already formed branches to form others. The peaks areas indicating branched chains were considerably small, being only about 1/10th of the peak areas of neighboring linear chains. Therefore, they were omitted from the following analysis. Further, large amounts of glucose were detected in all debranched samples. Since the levels of glucose were similar with and without GBE treatment (see Fig. S2), the glucose was unlikely to be caused by GBE enzyme activity and was thus excluded from the subsequent analysis.

As seen in Fig. 2, GBE modification of the four distinct starches resulted in products with highly similar chain length distribution profiles, independent of the crystalline type, degree of branching and relative amount of short chains. The modified starches generally showed distribution profiles with two maxima, the first around at DP 6–7 and the second at about DP 10–11 which was in very good agreement with previous literature (Murakami et al., 2006; Palomo et al., 2011; Rumbak et al., 1991; Suzuki et al., 2015; Zhang et al., 2019b). Interestingly, the second maximum in the products from GH13 GBEs on wrinkled pea starch was replaced by a broad shoulder. An explanation for that behavior could be the lack of long chains in the substrate. For PmGBE1, the shoulder was even entirely missing for wrinkled pea and tulip starch and contained substantially lower amounts of chains $>$ DP 8 than obtained from potato and waxy corn starch. This could hint at a preference of PmGBE1 for longer chains at a lower amylose content (Gaenssle, Satyawan et al., 2021).

Wrinkled pea starch consistently gave substantially lower peak areas for all GBEs which was in agreement with the lower amount of populations observed (Fig. 1A). The difference between the peak areas obtained from wrinkled pea and the other starches seemed to be less pronounced for GH13 GBEs than for GH57 GBEs with the activity of RmGBE resulting in almost the same amount for all starches.

Since the high amylose content of wrinkled pea appeared to have a far larger effect on the amount of populations (Fig. 1A) than on the amount of chains (Fig. 2), it is possible that the enzymes introduced a proportionally high number of branches due to the low initial degree of branching of wrinkled pea, resulting in the same structural dimensions but higher density of branches. Another possibility is that GH13 GBEs were relatively active on wrinkled pea but a part of their product only became soluble, and thus visible, after debranching. This would deem wrinkled pea unsuitable for producing slowly digestible starches through enzyme modification as a lower solubility or non-gelatinized starches would substantially decrease enzymatic digestion (Baldwin et al., 2015).

Notably, modification of potato starch with TtGBE resulted in lower amounts of chains which could be partly caused by the lower ratio between short and long chains (DP 9–12/DP 13–25) of wrinkled pea and potato starch (Gaenssle, Satyawan et al., 2021).

Apart from the aforementioned differences, activity of all GBEs, independent of the GH family, resulted in almost the same levels and profiles of peak areas for the other three starches, indicating the same extent of modification irrespective of the structural differences between the substrates. Generally, the amount of chains in the products that were DP $\geq$ 10 followed the pattern of the degree of branching of the substrate (waxy corn $>$ tulip $>$ potato $>$ wrinkled pea, (Gaenssle, Satyawan et al., 2021) although the differences were only minor. A similar pattern could

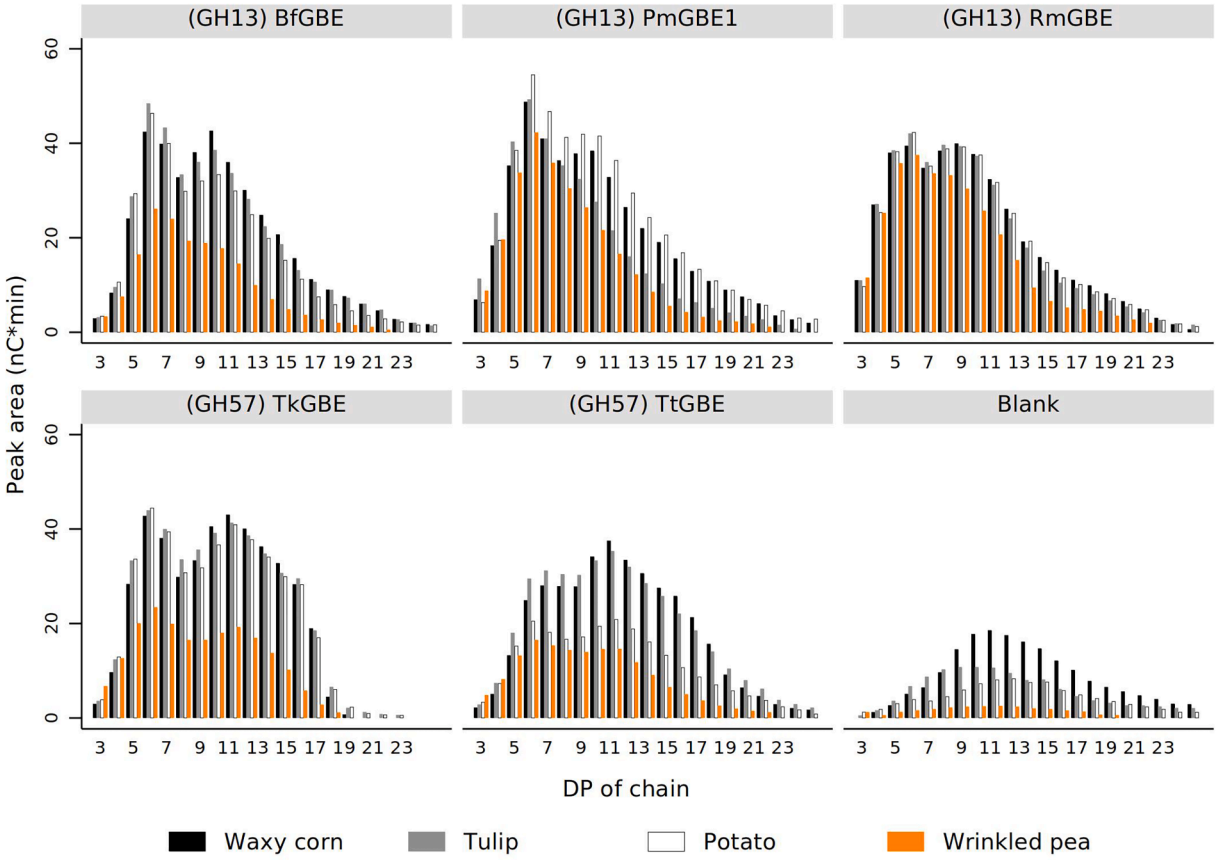

Fig. 2. Chain length distribution of four starches after modification for 24 h with five different branching enzymes. The enzyme reaction were conducted in triplicates and the analysis with HPAEC-PAD in duplicates and represented as mean (n = 2). The starch substrates are arranged from low to high amylose content as well as from high to low degree of branching.

not be observed for the shorter chains (DP < 10) and appeared to be more dependent on the enzyme. The shortest chains for which an increase was typically observed after enzyme modification were reported to be DP 4–6 (Murakami et al., 2006; Palomo et al., 2011; Rumbak et al., 1991; R. Suzuki et al., 2015) which were in agreement with the findings here although some fractions of DP 3, especially for RmGBE, were observed. Generally, GH57 GBEs showed a strong preference for chains shorter than DP 17 resulting in a pronounced drop in peak area between DP 16–18, especially for TkGBE. The product chain profiles of GH13 GBEs, on the other hand, showed smoother curves after the second peak which was in good agreement with literature (Sawada et al., 2014; Zhang et al., 2019b). However, GH13 GBEs tended to generate shorter chains (DP 3–10) while GH57 GBEs appeared to also produce longer chains (DP 11–16). The preference of GH13 GBEs for short branches and of GH57 GBEs for longer branches could explain the differences in the molecular sizes (Fig. 1A) of their products, even if the number of the introduced branches was the same. This could further imply that GH57 GBEs transfer their branches onto longer acceptor chains compared to GH13 GBEs.

A statistical analysis of the chain length fractions revealed significant correlations between the amount of fractions generated by the GBEs and the amylose content as well as the degree of branching of the substrate (Table 1). All GBEs, even when taken all together, showed a negative correlation with amylose content in the range of DP 9–24 with a Pearson's correlation coefficient from −0.958 for BfGBE (DP 9–12) to −0.773 for TtGBE (DP 9–12). Additionally, there were further some correlations detected for DP 6–8. The degree of branching indicated a positive correlation for the same fractions of chains, ranging from 0.723 for TkGBE (DP 13–24) to 0.893 for BfGBE (DP 9–12). Negative enzyme exhibited the highest Pearson's correlation coefficient with 0.991 and 0.966 for DP 9–12 and DP 13–24, respectively. Notably, the chain lengths of products obtained with PmGBE1 exhibited no correlation with the degree of branching of the substrates, supporting the patterns observed in Fig. 2. Since the substrates themselves exhibited the same behavior it can be speculated that the observed pattern might be more related to the fine structure of the substrate than with the enzyme activity. This is further supported by the fact that amylose consists of mostly long linear chains (about 3–5 chains of DP 270–525) while amylopectin is mainly composed of short branches (typically clusters of DP 18 chains) (Bertoft, 1991). Therefore, a high amylose content is positively correlated with chain length and negatively correlated with the degree of branching, matching the pattern observed here.

3.3. Chain length distribution of maltodextrin mixture

In addition to the starches, the GBEs were incubated with a mixture of linear maltodextrins (MD-Mix). Fig. 3 presents the chain length distribution of the substrate with and without debranching where the untreated sample is thought to only show linear (unbranched) chains and the debranched sample both linear and branched chains. Both samples showed evenly distributed amounts of chains over the entire detected range and a broader spectrum than the starch substrates (Fig. 2). From the untreated sample it was further clearly visible that the substrate did not only contain linear chains but also some branched chains and larger molecules as indicated by the small in-between peaks and the broad peak towards the end of the spectrum, respectively. This observation supported the results obtained from the size exclusion chromatography (Fig. 1B) with an estimated range of DP 6–90. Notably, the enzymatic debranching was more successful on the modified MD-Mix than the GBE treated starches, resulting in almost completely debranched samples (see Fig. S3), possibly indicating a more accessible structure due to the a lower density of chains compared to the modified starches.

The treatment of MD-Mix with GBEs enabled the estimation of the change in both linear and branched chains in response to GBE activity. The overall profiles of the generated chains (Fig. 4) roughly followed the same pattern as the modified starches (Fig. 2) although the maxima found on starches at DP 6 and DP 10–11 were both less pronounced and less consistent on MD-Mix. All GBEs increased the amount of chains in the range of DP 5–15 and reduced the number of chains above DP 15 which was in good agreement with literature (Roussel et al., 2013; Sawada et al., 2014). GH13 GBEs seemed to predominantly generate short branches, (about DP 3–9) while GH57 GBEs mainly produced branches longer than DP 8, matching the results obtained on the starches (Fig. 2).

Beside the chains, all GBEs were further found to cause the formation of substantial amounts of linear chains. These chains were likely byproducts from the branching activity or due to hydrolytic activity (Roussel et al., 2013) and were mainly of DP 5–12 (Fig. 4) with GH13 GBEs having a preference for DP 8–10 and GH57 GBEs for DP 6–7. Although almost no linear short chains were detected for starches (data not shown), these types of side products could also be formed there. However, for larger substrates it was more likely that these chain fragments were still attached to a larger molecule and thus remained indistinguishable from the branches.

Therefore, even though the maltodextrin substrate showed substantial differences to the starches in terms of size and chain length distribution, the products obtained after treatment with GBEs showed remarkable similarities to the modified starches.

3.4. Correlation between substrates and products

The analysis of the products obtained after GBE modification of four starchy substrates and a maltodextrin mixture revealed surprisingly few differences. The size and chain length distribution of the products were far more influenced by the enzyme type than by the structure of the substrates. The amount of the products acquired, however, were affected by the amylose content or the degree of branching of the substrate. This behavior hints at a highly versatile activity of GBEs that can be adapted to various substrate structures. Recently, GH13 enzymes have been found to be capable of α-1,4-transfer, elongating chains until they were

Table 1
Pearson correlation coefficients between the cumulative peak areas of chain length fractions of the products and the amylose content and branching of the starchy products.

	Amylose				**Branching**			
	DP 3–5	DP 6–8	DP 9–12	DP 13–24	DP 3–5	DP 6–8	DP 9–12	DP 13–24
BfGBE	−0.568	−0.841**	−0.958**	−0.942**	0.365	0.700*	0.893**	0.882**
PmGBE1	−0.043	−0.614	−0.778**	−0.797**	−0.054	0.354	0.603	0.655*
RmGBE	−0.321	−0.671*	−0.938**	−0.928**	0.345	0.449	0.786**	0.802**
TkGBE	−0.359	−0.884**	−0.941**	−0.881**	0.024	0.696	0.804**	0.723**
TtGBE	0.489	−0.664*	−0.773**	−0.804**	−0.669*	0.699	0.833**	0.834**
Blank	−0.423	−0.737**	−0.909**	−0.916**	0.108	0.734**	0.991**	0.966**
GH13	−0.125	−0.596**	−0.858**	−0.859**	0.051	0.433**	0.735**	0.751**
GH57	−0.012	−0.625**	−0.776**	−0.831**	−0.092	0.554**	0.739**	0.780**
All GBEs	−0.066	−0.491**	−0.807**	−0.803**	0.010	0.403**	0.727**	0.723**

** significant correlation (P ≤ 0.05) * tendency for significant correlation (0.05 < P ≤ 0.1), for exact levels of significance see Tables S1 and S2.

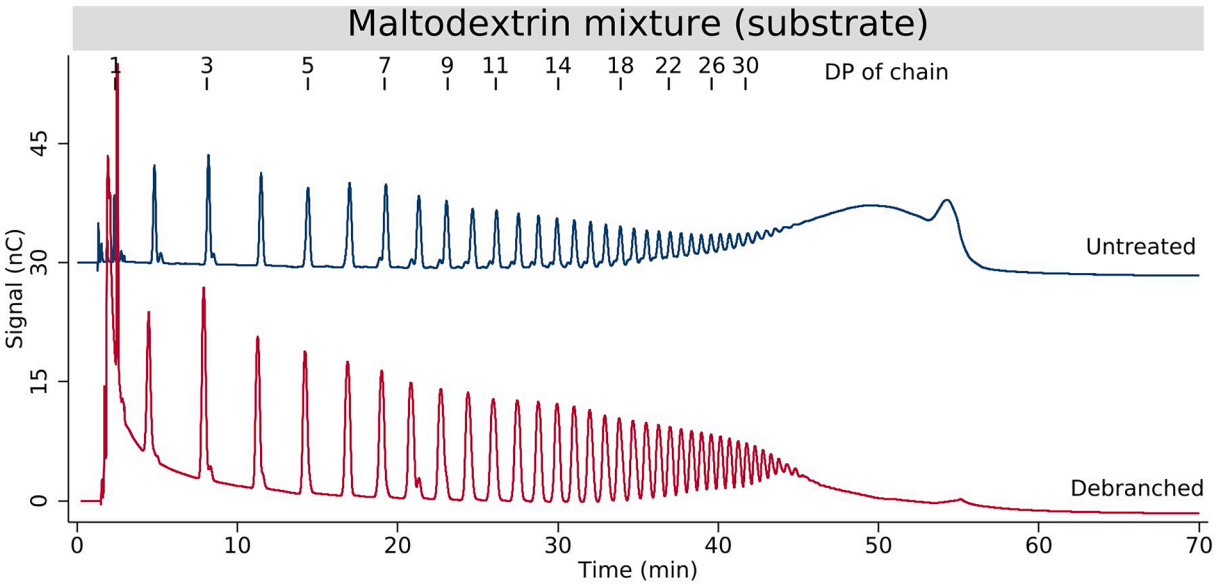

Fig. 3. Chain length distribution of the maltodextrin mixture (MD-Mix) before and after enzymatic debranching. The actual height of the glucose peak in the debranched sample was 137 nC. The branched sample indicates linear chains while in the debranched sample both linear chains and branches are visible.

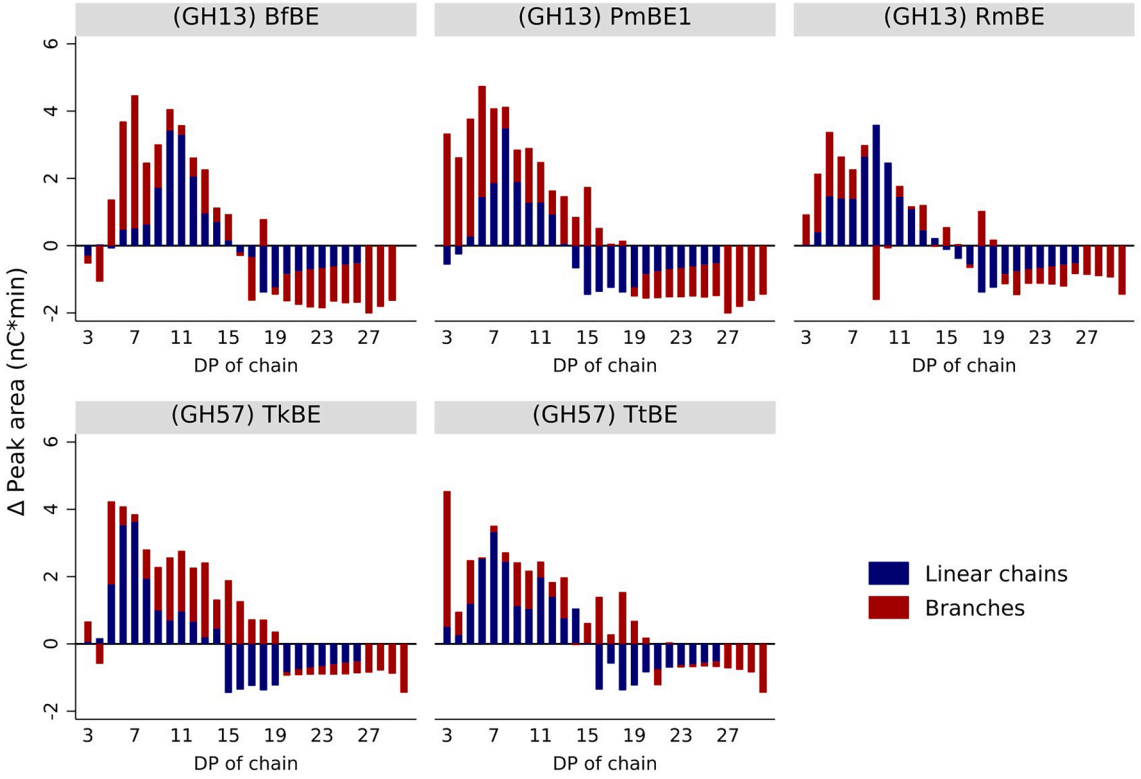

Fig. 4. Chain length distribution of maltodextrins after modification for 24 h of the maltodextin mixture (MD-Mix) with five branching enzymes. The products were analyzed with HPAEC-PAD with and without enzymatic debranching and chains were identified as followed: Linear chains (peak areas of branched samples) and branches (peak areas of debranched samples – linear chains). The values obtained without modification with branching enzyme (derived from Fig. 3) were subtracted from the modified products to display the change in peak area.

long enough for serving as a substrate for branching (Gaenssle, Bax, & van der Maarel, 2021; Roussel et al., 2013). Together with their ability to hydrolyze and branch chains (Yoon et al., 2008), GBEs could be able to modify a large array of substrate structures to a similar extent. GBEs have been reported to possess different levels of activity of distinct substrates (Zhang et al., 2019b) but they might not be detectable in this study since a long incubation length (24 h) was chosen to obtain final products. Since wrinkled pea gave consistently the lowest amounts of products (Fig. 1A and Fig. 2), it is possible that the amylose content is the rate determining step in the GBE modification.

In order to test this hypothesis, all products obtained for each enzymes were accumulated to calculate the ratio between the short and long chains (S_AF/LF ratio; DP 8–12 and DP $\geq$ 13, respectively). These values were plotted against three important characteristics of the

substrate (amylose content, degree of branching and S_AF/LF ratio) of which the Pearson's correlation coefficients are shown in Table 2. There was a significant positive correlation between the S_AF/LF ratio of the GBE treated starches and the amylose content of the starchy substrates, showing Pearson's correlation coefficients ranging from 0.885 to 0.948. Additionally, there was a significant negative correlation of the S_AF/LF ratio of the products with the degree of branching of the untreated starch (from −0.831 to −0.707). Notably, these correlations were also found for the S_AF/LF ratio of the untreated starch (Blank, Table 2), having coefficients of 0.752 and −0.602 for amylose and degree of branching, respectively, but GBE treatment increased the level of correlation, especially regarding the amylose content. Overall, a high amylose content and a low degree of branching resulted in a high accumulation of short chains. Thus, a large amount of long linear chains resulted in a higher relative number of short branches. Since only the S_AF/LF ratio while the total peak areas decreased (Fig. 2), a low amylose content appeared to lead to a higher number of total chains which contained a lower percentage of short chains. It further suggests that the studied GBEs were more active on samples with a lower amylose content and chains longer than DP 12. It should be noted that the actual S_AF/LF ratios might be lower than estimated as the signal response (nC/μg α-glucan) of the HPAEC-PAD decreased sharply between DP 1–7 before leveling out around DP 15 but is unlikely to differ substantially (Koch et al., 1998).

In contrast to the amylose content and the degree of branching, no significant correlations could be detected between the S_AF/LF ratio of the products and the starchy substrates (Table 2). This showed that the chain length distribution of the substrate did not govern the relative amount of short chains in the product, possibly by using the inner chains (stretches between two branch points) as their donor chains as has been reported for two bacterial GBEs (from *E. coli* and *Synechococcus elongatus*) (Sawada et al., 2014).

In this study, the products were analyzed regarding their population size and their chain length distribution. However, it is possible that other structural features of the substrate may strongly influence the product such as the degree of branching or the internal chain length. The degree of branching was not determined due to the incomplete debranching (Fig. S1) and the high accumulation of glucose (Fig. S2) in the products which would result in highly inaccurate estimations. A previous study on the modification of samples with varying amylose/amylopectin ratio indicated some correlation between the degree of branching and amylose content (Zhang et al., 2019b). In starches, the internal chain length is positively correlated to the average chain length (Bertoft et al., 2008). Since the amount of chains produced during GBE treatment was correlated to the amylose content of substrates, the internal chain length might be as well.

GBEs can be applied to generate highly branched starches which are less susceptible to enzymatic digestion (Li et al., 2017; van der Maarel et al., 2010). Even though the type of substrate appears to be of little consequence to the structure of the product obtained through GBE modification, it might play a role in the digestibility of the products. The effect of GBE treatment on digestibility has not been studied in this thesis. Therefore, this would be an important feature for further investigation. Considering the positive correlation between the degree of branching and the digestibility of the native starches (Gaenssle, Satyawan et al., 2021) there might be a similar correlation for the modified starches. This is supported by the observation that extended treatment of cassava starch with GBE from *Geobacillus thermoglucosidans* led to a gradually increased resistance to digestibility (Li et al., 2017). If, however, the substrate type does not influence the rate of enzymatic digestion, the substrate can be selected based on its structural features. Native starches can vary significantly between botanical sources in their fine structure. One of the characteristics is the amylose content (Gaenssle, Satyawan et al., 2021). Amylose is not only relatively insoluble in aqueous solutions (Green et al., 1975) but also prone to retrogradation (Greenwood & Hourston, 1971). Therefore, a low amylose content simplifies the preparation of the sample and assures that the entire sample is subjected to enzyme treatment. Furthermore, substrates could be used that are more readily available and easy to produce, simplifying the process even more.

Table 2
Pearson correlation between the S_AF/LF ratio[a] of the GBE products and three properties of the starchy substrates.

	Amylose	Branching	S_AF/LF ratio[a]
BfGBE	0.942*	−0.831*	−0.589
PmGBE1	0.921*	−0.772*	0.022
RmGBE	0.948*	−0.805*	−0.269
TkGBE	0.884*	−0.707*	−0.319
TtGBE	0.859*	−0.778*	−0.518
Blank	0.752*	−0.602	−0.092
GH13	0.921*	−0.788*	−0.244
GH57	0.807*	−0.687*	−0.388
all GBEs	0.811*	−0.689*	−0.260

* Significant correlation ($P \leq 0.05$); [a] Ratio between short (DP 8–12) and long (DP ≥ 13) chains, for exact levels of significance see Table S3.

4. Conclusion

Five branching enzymes (BfGBE, PmGBE1, RmGBE, TkGBE and TtGBE) have been applied to modify four starches (potato, tulip, wrinkled pea and waxy corn starch) and a mixture of linear maltodextrins. The obtained products were predominantly influenced by the type of GBE and further by the amylose content and the degree of branching of the substrates. GH57 GBEs generated populations of larger size from starches (about 100–700 kDa) than GH13 GBEs (~6–150 kDa) and the size of the product showed correlation with the degree of branching (positive) and amylose content (negative) of the substrate whereas the GH13 GBEs exhibited the opposite behavior. The amylose content of the substrate had further a negative effect on the amount of products and subsequent amount of chains obtained. Analysis of the products obtained from GBE modification of the maltodextrin mixture revealed that GH57 GBEs generated larger branches (DP 11–16) than GH13 GBEs (DP 3–10).

CRediT authorship contribution statement

Aline L.O. Gaenssle: Formal analysis, Methodology, Visualization, Writing – original draft. **Marc J.E.C. van der Maarel:** Funding acquisition, Resources, Supervision. **Edita Jurak:** Conceptualization, Project administration, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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