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The influence of α-1,4-glucan substrates on 4,6-α-D-glucanotransferase reaction dynamics during isomalto/malto-polysaccharide synthesis

C.E. Klostermann [a,b], P.H. van der Zaal [a], H.A. Schols [b], P.L. Buwalda [a,c], J.H. Bitter [a,*]

[a] Biobased Chemistry & Technology, Wageningen University & Research, PO Box 17, 6700 AA Wageningen, the Netherlands
[b] Laboratory of Food Chemistry, Wageningen University & Research, P.O. Box 17, 6700 AA Wageningen, the Netherlands
[c] Coöperatie AVEBE u.a., P.O. Box 15, 9640, AA, Veendam, the Netherlands

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ABSTRACT

Starch-based isomalto/malto-polysaccharides (IMMPs) are soluble dietary fibres produced by the incubation of α-(1 → 4) linked glucans with the 4,6-α-glucanotransferase (GTFB) enzyme. In this study, we investigated the reaction dynamics of the GTFB enzyme by using isoamylase debranched starches as simplified linear substrates. Modification of α-glucans by GTFB was investigated over time and analysed with ^{1}H NMR, HPSEC, HPAEC combined with glucose release measurements. We demonstrate that GTFB modification of linear substrates followed a substrate/acceptor model, in which α-(1 → 4) linked glucans DP ≥ 6 functioned as donor substrate, and α-(1 → 4) linked malto-oligomers DP < 6 functioned as acceptor. The presence of α-(1 → 4) linked malto-oligomers DP < 6 resulted in higher GTFB transferase activity, while their absence resulted in higher GTFB hydrolytic activity. The information obtained in this study provides a better insight into GTFB reaction dynamics and will be useful for α-glucan selection for the targeted synthesis of IMMPs in the future.

1. Introduction

Isomalto/malto-polysaccharides (IMMPs) are α-glucans produced by the enzymatic modification of starch with 4,6-α-glucanotransferase (GTFB) [1]. Modification of starch with the GTFB enzyme results in an increase of α-(1 → 6) glycosidic linkages and a decrease of easily digestible α-(1 → 4) glycosidic linkages [2]. IMMPs are therefore considered a dietary fibre [1] and previous research highlights their possible prebiotic potential [3,4]. In addition, it was shown recently that oligomers produced by GTFB had immune-modulatory effects [5]. In order to get a better understanding on IMMP's functionality, it is essential to understand how the GTFB enzyme interacts with its substrate during α-glucan modification.

In previous research, the activity of the GTFB enzyme was investigated on monodisperse low Mw model substrates, such as maltoheptaose [5,6]. GTFB activity on maltoheptaose led to the formation of compounds both smaller and larger than the initial substrate. This showed that the GTFB enzyme is able to polymerise α-glucans by elongating linear α-(1 → 6) linked glycosidic residues inside the acceptor molecule at the expense of α-(1 → 4) linked glycosidic residues in the donor molecule [6]. This disproportionating effect is also observed for an amylomaltase (4-α-glucanotransferase) found in *Pyrobaculum aerophilum* [7,8] and some glucansucrases [9]. Experiments with monodisperse α-glucans, which are expensive and rare, are not easily transferable to technical applications. To make technical applications possible, research is needed using an easily obtainable source such as starch. GTFB activity has previously been studied on native starches [1,10,11]. GTFB modification of several starches showed the formation of large α-(1 → 6) linked glycosidic chains, especially in the presence of amylose [11]. It was shown that the GTFB enzyme has a preference for linear α-(1 → 4) linked substrates as donor molecules, while α-(1 → 4,6) linked branching points were limiting its performance [1,12]. However, starches generally have a high polydispersity index [13] and vary in degree of branching and amylose content depending on the botanical source [14,15]. Although the use of starch is a lot more realistic in an industrial scenario, the complexity of the substrate made it still hard to verify the underlying mechanism of the complex enzymatic disproportionation reaction.

To study the reaction mechanism of GTFB, a substrate in between simple monodisperse α-glucans and overcomplex native starch would be highly beneficial. Debranched amylopectin seems to be an ideal substrate, since it was shown that especially linear α-(1 → 4) linked amylose substrates were modified by the GTFB enzyme [11]. While a debranched α-glucan substrate still has high polydispersity [16], its chain length distribution (CLD) can be controlled by selecting the origin of the starch that is debranched.

In this study, we selected debranched amylomaltase treated potato starch (ATPS) for its broad CLD and debranched waxy potato starch (WPS) for its narrower CLD. The effect of a broad and narrow CLD on

* Corresponding author.
E-mail address: harry.bitter@wur.nl (J.H. Bitter).

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GTFB activity was investigated over time and analysed with HPSEC, HPAEC, ^{1}H NMR and enzymatic fingerprinting. The use of simplified substrates in combination with analytical read-outs allowed for an in-depth investigation on the limits of GTFB transferase activity.

2. Materials & methods

2.1. Materials

Waxy potato starch (WPS, Eliane 100) and amylomaltase treated potato starch (ATPS, Etenia 457) were provided by Avebe (Veendam, the Netherlands). Dextran (~35 kDa), glucose, maltotriose and isomaltotriose were obtained from Sigma-Aldrich (St. Louis, MO, USA). Isomaltose was obtained from Megazyme (Bray, Wicklow, Ireland) and maltose was obtained from Fluka (Buchs, Switzerland). Isoamylase (EC 3.2.1.68) (*Pseudomonas* sp.), isopullulanase (EC 3.2.1.57) (*Aspergillus niger*) and β-amylase (EC 3.2.1.2) (Barley) were purchased from Megazyme. Dextranase (EC 3.2.1.11) (*Chaetomium erraticum*) was obtained from Sigma-Aldrich.

2.2. GTFB-ΔN production and activity

4,6-α-glucanotransferase-ΔN (GTFB-ΔN) was produced and purified according to van der Zaal et al. (2018). GTFB-ΔN activity was measured with a GOPOD assay (Megazyme) and was comparable to previous research [10,12].

2.3. IMMP synthesis from linear α-(1 → 4) linked glucans

WPS or ATPS was suspended at 2.5 mg/mL in 20 mM acetate buffer, pH = 5.0 containing 5 mM $CaCl_2$. The suspension was autoclaved at 121 °C for 15 min and cooled to 40 °C. The solubilised starch was debranched by adding isoamylase (0.16 U/mg substrate) and incubating in a Kuhnershaker Climo-shaker IFF1-X (Kuhner, Bisfelden, Switzerland) at 40 °C and 100 rpm for 17 h. The isoamylase was inactivated in a water bath at 95 °C for 15 min and the samples were subsequently cooled to 37 °C. IMMP synthesis was carried out by adding 0.3 mg GTFB-ΔN/g substrate and incubating at 37 °C for 0, 7, 24, 48 and 70 h. After the modification, GTFB-ΔN was inactivated in a water bath at 95 °C for 15 min. Samples were stored at room temperature to avoid crystallisation and analysed within 3 days after preparation.

2.4. Enzymatic fingerprinting assay

IMMPs synthesized from debranched ATPS were further analysed with an enzymatic fingerprinting method adapted from van der Zaal et al. (2019) [11]. The sample solutions (2.5 mg/mL) were incubated with different hydrolytic enzymes in a 20 mM acetate buffer, pH = 5.0 containing 5 mM $CaCl_2$. β-amylase and isopullulanase were added at concentrations of 0.16 U/mg substrate and dextranase was added at a concentration of 0.052 U/mg substrate. The following incubations were performed: β-amylase, isopullulanase, dextranase individually, and two one-pot incubations of β-amylase with isopullulanase and β-amylase with dextranase. All incubations were performed in a Kuhnershaker at 39 °C and 100 rpm for 4 h. After the reaction, the enzymes were inactivated in a water bath at 95 °C for 15 min. Reference samples containing α-(1 → 6) and α-(1 → 4) linked oligomers were prepared in similar fashion by incubating dextran (2.5 mg/mL) with dextranase (0.052 U/mg) and by incubating ATPS (2.5 mg/mL) with isoamylase (0.16 U/mg) respectively (Supplementary Fig. S1).

2.5. ^{1}H NMR spectroscopy

The total α-(1 → 6) content and amount of α- and β-reducing ends were measured with ^{1}H NMR spectroscopy adapted from van der Zaal (2018) [12]. Freeze-dried IMMP was exchanged once with D_2O by lyophilisation and dissolved in D_2O (99.9 atom % D, Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 10 mg/mL. Samples were shaken and heated up to 70 °C in a Thermomixer (Eppendorf, Hamburg, Germany) to ensure maximal solubility during the 1H NMR measurement. 1D ^{1}H NMR spectra were recorded at 70 °C on a Bruker Avance 600 spectrometer equipped with a cryo-probe (Billerica, MA, USA) located at MAGNEFY (MAGNEtic resonance research FacilitY) in Wageningen, the Netherlands. Structures were characterized using the ^{1}H NMR structural-reporter-group concept for α-D-glucans [6,17].

2.6. Determination of molecular weight distribution by HPSEC-RI

Sample solutions (2.5 mg/mL) were centrifuged at 7317 ×*g* and 20 °C for 10 min and the supernatant was used for HPSEC analysis. An Ultimate 3000 HPLC (Dionex, Sunnyvale, USA) was used with a column set which consisted of three in series connected TosoHaas (Tokyo, Japan) TSK-Gel columns (4000PWXL-3000PWXL-2500PWXL), (6 × 150 mm), with a guard column and a Shodex type RI-101 refractive index detector (Showa Denko, K.K., Kawasaki, Japan). The eluent was 0.2 M NaNO3 and elution was performed with a flow of 0.6 mL/min at 55 °C. A volume of 10 μL sample solution was injected onto the column. A pullulan standard series (180–780,000 Da) (Fluka) was used for calibration. The buffer and all enzymes were run separately as controls. Data analysis was performed with Chromeleon 7.1 software (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

2.7. Determination of mono- and oligomers by HPAEC-PAD

Sample solutions were diluted to 0.5 mg/mL with Millipore water and centrifuged at 7317 ×*g* and 20 °C for 10 min, the supernatant was used for HPAEC analysis. The analysis was performed on an ICS5000 High Performance Anion Exchange Chromatography system with Pulsed Amperometric Detection (HPAEC-PAD) (Dionex) equipped with a CarboPac PA-1 column (2 × 250 mm) and a CarboPac PA-1 guard column (2 × 25 mm). The two mobile phases were (A) 0.1 M NaOH and (B) 1 M NaOAc in 0.1 M NaOH and the flow rate was set to 0.3 mL/min. The following gradient was used: 0–50 min 5–40% B, 50–65 min 40–100% B, 65–70 min 100% B ending with a 70–85 min re-equilibration at 5% B. A volume of 10 μL sample solution was injected onto the column. Glucose, maltose, maltotriose, isomaltose and isomaltotriose (10–100 μg/mL) were run as standards, together with dextranase treated dextran as α-(1 → 6) linked oligomer reference and isoamylase debranched ATPS as α-(1 → 4) linked oligomer reference. Data analysis was performed with Chromeleon 7.1 software (Thermo Fisher Scientific).

2.8. Free glucose determination

Free glucose was measured with the GOPOD assay (Megazyme), the preparation of the GOPOD assay was performed according to the supplier's manual. The inactivated samples (50 μL) were mixed with 1.5 mL of GOPOD reagents and incubated in a Thermomixer (Eppendorf) at 40 °C and 450 rpm for 20 mins. The absorbance at 510 nm was measured with a DU 720 UV/VIS spectrophotometer (Beckman Coulter, Brea, CA, USA).

3. Result & discussion

3.1. Substrate chain length distribution

Isoamylase was used to debranch amylomaltase treated potato starch (ATPS) and waxy potato starch (WPS). The chain length distribution (CLD) of debranched ATPS (dATPS) and debranched WPS (dWPS) is displayed in Fig. 1. The HPAEC profile of dWPS shows that the smallest chain present is DP 6, whereas dATPS also contains smaller oligomers, eluting before RT = 15 min (Fig. 1). HPSEC profiles show that dATPS

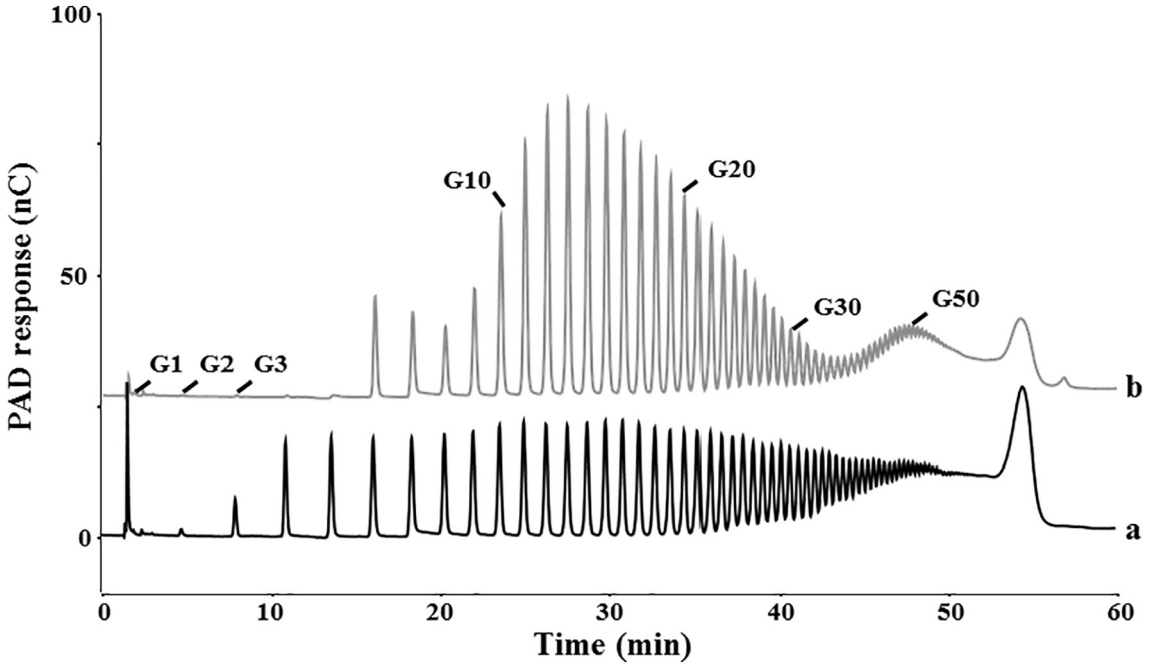

Fig. 1. HPAEC profile of debranched amylomaltase treated potato starch (dATPS) (a) and debranched waxy potato starch (dWPS) (b). The elution times of the HPAEC standards; glucose, maltose, maltotriose are indicated as G1, G2, G3 respectively.

contains more high molecular weight glucans compared to dWPS (see below). The presence of smaller and larger oligomers in dATPS compared to dWPS is caused by the disproportionating effect of amylomaltase treatment resulting in a broader CLD (Ayudhaya et al., 2016). Both substrates consist solely of α-(1 → 4) linked glucans and vary only in their respective CLDs. Since α-(1 → 4) linked glucans are considered to be the best known substrate for the GTFB-ΔN enzyme [1,12,18], both substrates should be perfectly suited for the enzymatic modification by GTFB-ΔN.

3.2. Transferase activity of GTFB-ΔN is preceded by initial hydrolytic activity

The substrates dATPS and dWPS were incubated with the GTFB-ΔN enzyme for 0, 7, 24, 48 or 70 h. Previous research has shown that the GTFB enzyme displays both hydrolytic and transferase activity, with the hydrolytic activity splitting α-(1 → 4) glycosidic linkages and the transferase activity forming α-(1 → 6) glycosidic linkages from the reassembly of the released glucose still present in the active site of the enzyme, to a donor molecule [18]. GTFB-ΔN hydrolytic activity was monitored by tracking both the percentage of reducing ends and free glucose released and GTFB-ΔN transferase activity was monitored by tracking the percentage of α-(1 → 6) linked glycosidic linkages formed (Fig. 2). Fig. 2 shows that GTFB-ΔN transferase activity was slower than initial GTFB-ΔN hydrolytic activity, since the amount of reducing ends and the amount of free glucose initially increased faster than the total amount of α-(1 → 6) glycosidic linkages. The lag phase for transferase activity was shorter for dATPS compared to dWPS resulting in dATPS scoring consistently higher in total α-(1 → 6) glycosidic linkage content, amount of free glucose and the amount of reducing ends (Fig. 2). The percentage α-(1 → 6) linked glycosidic linkages after 70 h of GTFB-ΔN incubation was 69% for dATPS and 63% for dWPS. These initial values indicate that higher rates of GTFB-ΔN transferase activity were preceded by initial hydrolytic activity, even in purely linear samples.

3.3. GTFB-ΔN synthesizes IMMPs that have a larger mw compared to the initial substrate

GTFB-ΔN modification of dATPS and dWPS was investigated in further detail with HPSEC and HPAEC (Fig. 3). HPSEC profiles show that

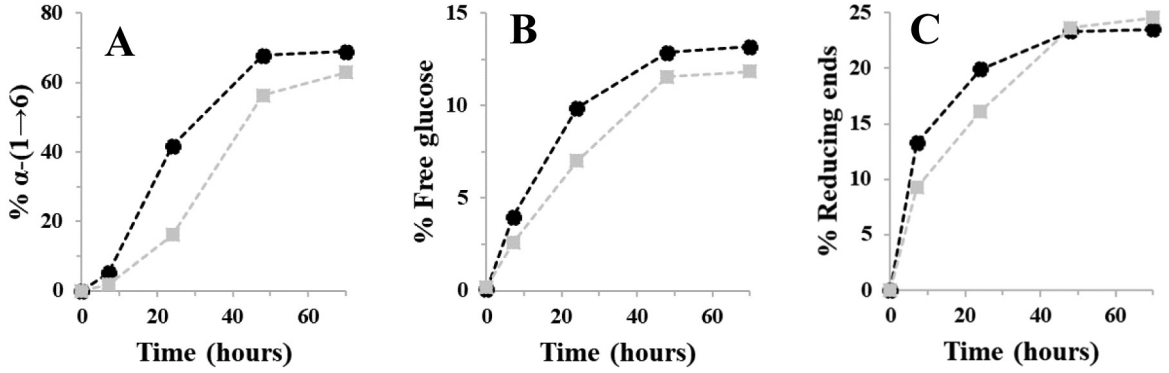

Fig. 2. Reaction of debranched amylomaltase treated potato starch (dATPS) (●) and debranched waxy potato starch (dWPS) (■) with the GTFB-ΔN enzyme over time. The figures represent the content of α-(1 → 6) linked glycosidic linkages of total linkages (A); free glucose (B) and reducing ends (C) and are expressed as percentage of the total amount of free and bound glucose present in the sample.

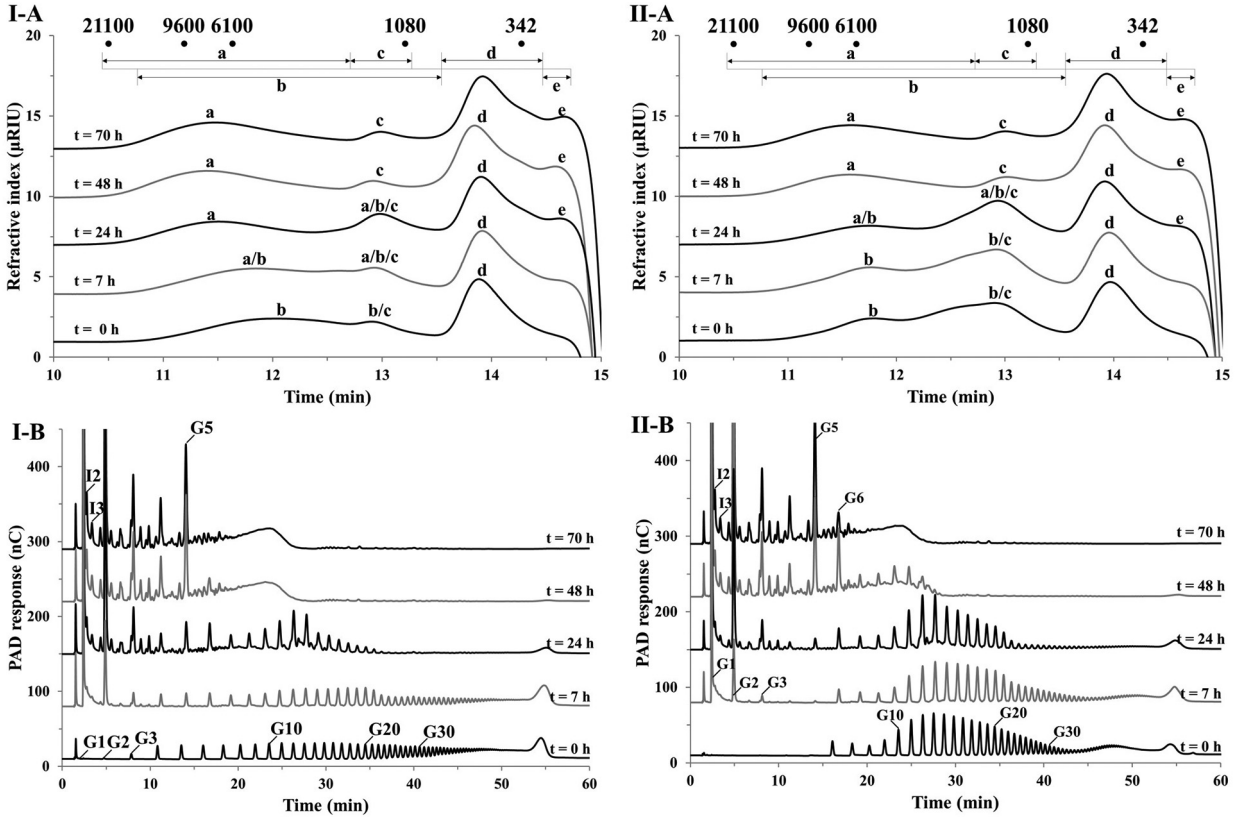

Fig. 3. HPSEC (A) and HPAEC (B) profiles of I: isoamylase debranched amylomaltase treated potato starch (dATPS) and II: isoamylase debranched waxy potato starch (dWPS), incubated with GTFB-ΔN for 0, 7, 24, 48 and 70 h. The Mw (Da) of the pullulan standards are indicated (●). The letters represent molecules eluting from the SEC column at the respective retention times; a: IMMPs, b: linear α-(1 → 4) glucans, c: GTFB-ΔN enzyme, d: buffer/oligomers, e: released glucose. The HPAEC elution times of glucose, maltose, maltotriose, isomaltose and isomaltotriose are indicated as **G1**, **G2**, **G3**, **I2** and **I3** respectively.

both dATPS and dWPS gained a higher molecular weight (Mw) and a broader Mw distribution after GTFB-ΔN modification, with a Mw distribution that contained both larger and smaller populations than the initial substrate (Fig. 3A). Although we observe high polydispersity in all samples, the largest population of dATPS showed an average size increase from 5.8 kDa to 9.5 kDa and the largest population of dWPS showed an average size increase from 3.2 kDa to 6.8 kDa after 70 h of incubation with GTFB-ΔN. This indicates that GTFB-ΔN was able to polymerise α-glucans (Fig. 3A), despite a considerable increase in the amount of free glucose and small oligomers (Fig. 2).

HPAEC profiles of both samples show a transition from a majority α-(1 → 4) glycosidic linked substrate to a majority α-(1 → 6) glycosidic linked product during the 70 h incubation (Fig. 3B, panel I and II). This transition is clearly visible, since α-(1 → 6) linked oligomers elute faster than α-(1 → 4) linked oligomers [11], (Supplementary Fig. S1). The transition of α-(1 → 4) linked glycosidic substrate (Fig. 3B: t = 0 (G2, G3, G10, G20)) into α-(1 → 6) linked glycosidic product was followed by monitoring the disappearance of the homogenous series of α-(1 → 4) oligomers (Fig. 3B: t = 0 to t = 24), as well as by the appearance of the 'hump' of majority α-(1 → 6) linked glucans that is eluting between 20 and 25 min (Fig. 3B: t = 24 to t = 70).

After 48 h for dATPS and 70 h for dWPS, only five α-(1 → 4) linked peaks were left in the reaction mixture (Fig. 3B). Most of these peaks were not originally present in the substrate and were formed as by-product of GTFB hydrolytic and transferase activity. These leftover α-(1 → 4) oligomers (DP < 6) eluting before RT = 15 min (Fig. 3B: dATPS (t = 48 h and 70 h), dWPS (t = 70 h)), clearly indicate the limits of what the GTFB-ΔN enzyme is still able to recognize and use as a donor substrate. This apparent minimum substrate length (DP = 6) coincides with the minimum length that is needed to form a single α-(1 → 4) glycosidic helix [19]. The α-(1 → 4) linked oligomers with a DP < 6 are obviously not accepted as donor substrate by the carbohydrate binding module (CBM) of the GTFB-ΔN enzyme. After 70 h of incubation, there were peaks that deviated from the α-(1 → 6) linked and α-(1 → 4) linked reference samples (Fig. 3B, Supplementary Fig. S1). These components were identified previously as hybrid molecules consisting of both an α-(1 → 6) linked segment and an α-(1 → 4) linked segment [11].

The increase of glucose (G1), maltose (G2) and maltotriose (G3) after 7 h of reaction time, indicates a mostly hydrolytic GTFB-ΔN activity at the start of the reaction (Fig. 3B). The increase in linear α-(1 → 4) linked glycosidic oligomers, such as maltose and maltotriose, may confirm that the GTFB-ΔN enzyme was indeed capable of *endo*-activity as demonstrated in previous research [18].

The change in chain length distribution during GTFB-ΔN modification shows that a majority of α-(1 → 4) linked glucans were converted into α-(1 → 6) linked glucans. The composition at the end of the reaction reveals that only α-(1 → 4) linked glucans DP ≥ 6 were a suitable donor substrate for GTFB-ΔN transferase activity. The slower transferase activity of GTFB-ΔN compared to its hydrolytic activity indicates that the α-(1 → 4) linked oligomers (DP < 6) created by GTFB-ΔN hydrolysis can potentially function as acceptors for GTFB transferase activity (Figs. 2 and 3). These acceptors, in turn, will increase the rate of the GTFB-ΔN transferase activity, since previous research has shown that GTFB amylose degradation increased after the addition of low DP glycosidic acceptors [10].

3.4. Enzymatic fingerprinting reveals IMMPs consisting of at least 130 α-(1 → 6) linked residues

The modified molecules after treatment with the GTFB-Δ-N enzymes were characterized using an enzymatic fingerprinting method

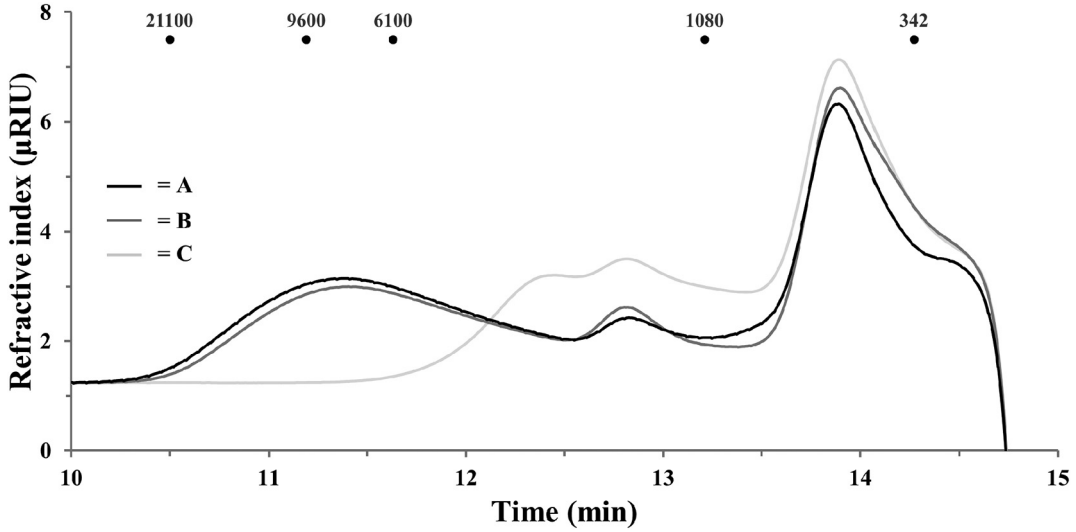

Fig. 4. HPSEC profile of debranched amylomaltase treated potato starch after 70 h of incubation with the GTFB-ΔN enzyme before (dATPS-70 h —, line A), and after hydrolysis by a isopullulanase and β-amylase mixture (—, line B) or by a dextranase and β-amylase mixture (—, line C). The peak eluting at retention time t = 14 min corresponds to the buffer. The Mw (Da) of the pullulan standards are indicated (●).

adapted from van der Zaal et al. (2019) [11] as exemplified here for dATPS. This approach was used in order to confirm the presence of hybrid molecules and to get more information on the size of the linear α-(1 → 6) linked glucans produced by the GTFB-ΔN enzyme. Analysis of the reaction products confirmed the preferred substrate and acceptors of the GTFB-ΔN enzyme and even provided more insights in the mechanism involved. HPSEC profiles of 70 h GTFB-ΔN treated dATPS (dATPS-70 h) and the corresponding hydrolysed samples are displayed in Fig. 4. All α-(1 → 4) glycosidic linkages present in dATPS-70 h sample (Fig. 4, line A) were hydrolysed to maltose or maltotriose by a one-pot incubation with isopullulanase and β-amylase (Fig. 4, line B). The slight decrease in size indicates that most α-(1 → 6) linked glucans contained a small amount of α-(1 → 4) linked oligomers, but that most of the high molecular weight (HMW) fraction in dATPS-70 h consisted of α-(1 → 6) linked glucans. When comparing the HPSEC profiles with the pullulan standards, it is observed that GTFB-ΔN was able to synthesize linear α-(1 → 6) linked glucans up to 21 kDa or approximately DP 130, which is slightly higher than previously reported [11]. The one-pot incubation with the *endo*-enzyme dextranase and *exo*-enzyme β-amylase resulted in hydrolysis of the α-(1 → 6) linked glucans to α-(1 → 6) linked oligomers and hydrolysis of the leftover solely α-(1 → 4) linked oligomers, resulting in a decrease in Mw (Fig. 4, line C). In this figure, the large decrease in the HMW fraction confirms that the HMW fraction of dATPS-70 h indeed consisted mostly of α-(1 → 6) linked glucan polymers.

Different acceptors of GTFB-ΔN transferase activity were identified by HPAEC analysis of dATPS-70 h before and after incubation with isopullulanase (Fig. 5). Following the scheme depicted in Fig. 6, the capability of maltose, maltotriose, maltotetraose, maltopentaose and maltohexaose as acceptors could possibly be proven by the respective increase in glucose (**G1**), maltose (G**2**), maltotriose (**G3**), maltotetraose (**G4**) and maltopentaose (**G5**). HPAEC chromatography of dATPS-70 h after incubation with isopullulanase shows an increase of especially maltotriose and maltotetraose (Fig. 5). The isopullulanase incubation

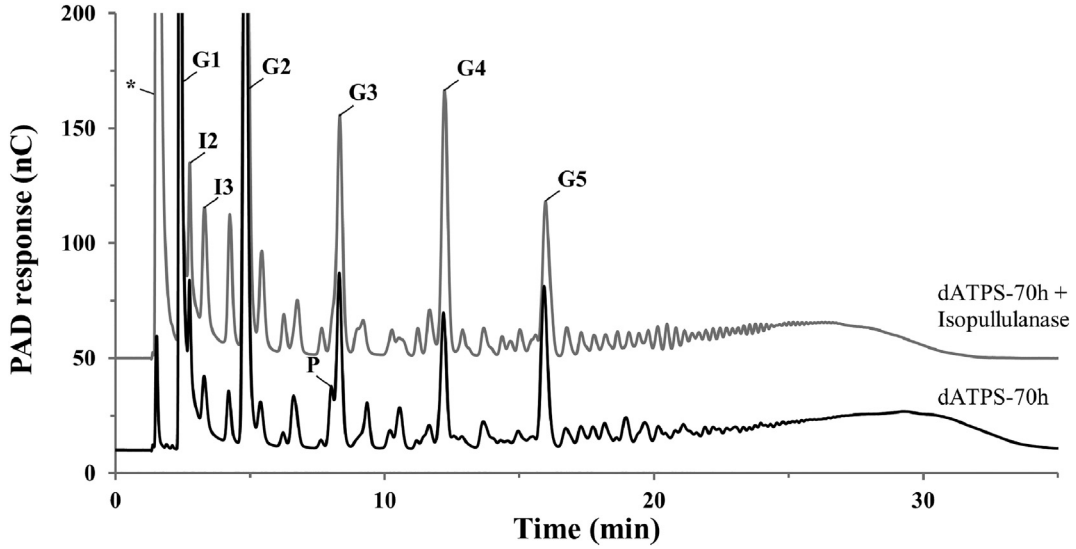

Fig. 5. HPAEC profile of debranched amylomaltase treated potato starch (dATPS) after 70 h of incubation with the GTFB-ΔN enzyme (dATPS-70 h, —), hydrolysed with isopullulanase (dATPS-70 h + Isopullulanase, —). Indicated are; glucose (**G1**), maltose (**G2**), maltotriose (**G3**), maltotetraose (**G4**), maltopentaose (**G5**), isomaltose (**I2**) and isomaltotriose (**I3**). The elution time of panose (P) was derived from [20]. The glycerol peak (*) originated from the enzyme solution.

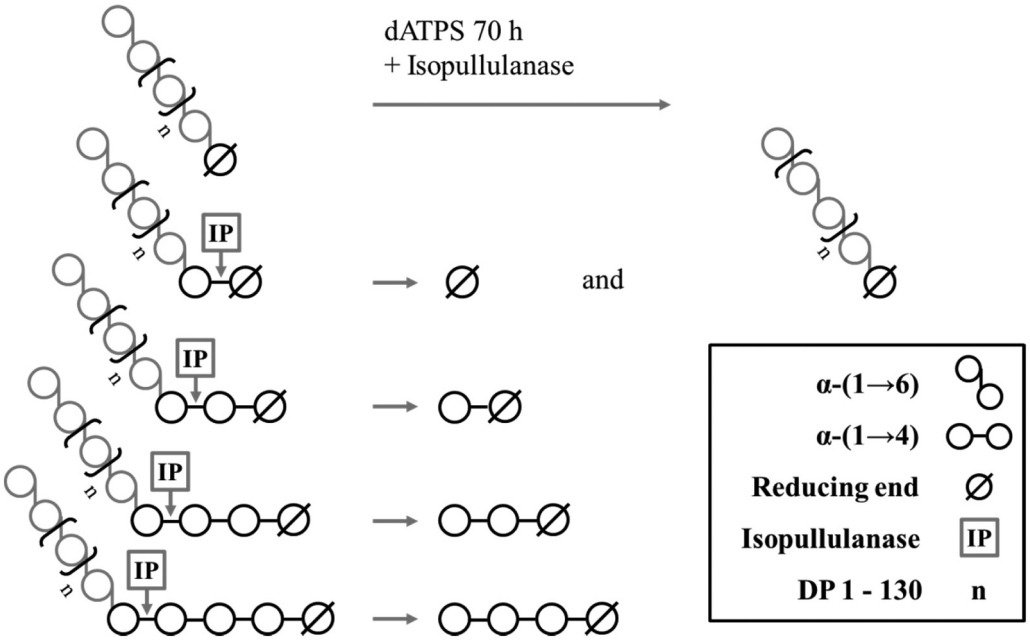

Fig. 6. Proposed IMMP structures produced by GTFB-ΔN activity on linear α-(1 → 4) linked glucans, based on the enzymatic fingerprinting with isopullulanase of dATPS-70 h and analysis of oligomers present in the hydrolysed sample.

thus indicates that especially maltotetraose and maltopentaose are relatively good acceptors for GTFB-ΔN transferase activity. The increase in maltopentaose after isopullulanase incubation was minimal, meaning that we cannot really confirm the existence of maltohexaose acceptors for GTFB-ΔN transferase activity (Fig. 5), although maltohexaose was identified as an acceptor for GTFB-ΔN transferase activity in previous research [6]. Our results do not show a clear increase of glucose and maltose after isopullulanase treatment. However, analysis of another IMMP sample produced in a similar way showed a clear increase of glucose and maltose after isopullulanase treatment, indicating that maltose and maltotriose are good acceptors for GTFB-ΔN too (data not shown). In addition, the presence of panose in the dATPS-70 h sample confirms the existence of maltose as acceptor for GTFB-ΔN transferase activity (Figs. 5 and 6). The presence of isomaltose and isomaltotriose in the dATPS-70 h sample proves the existence of glucose as acceptor (Figs. 5 and 6).

Next to an increase in intensity of α-(1 → 4) linked oligomers due to isopullulanase treatment, the HPAEC profile also shows the α-(1 → 6) linked glucans (Fig. 5). When looking carefully to the chromatogram, one can see that the intensity of the α-(1 → 6) linked glucan oligomers peaks is increasing and decreasing over retention times (e.g. 17–22 min and 22–24 min), creating a wave-like pattern. This same wave-like pattern of the α-(1 → 6) linked glucans was observed after incubation of another IMMP sample with isopullulanase (data not shown). Because of this observation, we hypothesize that the length of the α-(1 → 6) linked chain created by GTFB-ΔN depends on the length of the α-(1 → 4) linked acceptor. The combination of enzymatic fingerprinting and chromatography made this study the first that was able to extensively characterize and prove the existence of high DP IMMP structures depicted in Fig. 6.

3.5. GTFB-ΔN activity is dependent on presence of small acceptor molecules

Comparison of dATPS and dWPS over time (Fig. 3) shows that a substrate without initial DP < 6 oligomers resulted in slower and less GTFB-ΔN transferase activity. The GTFB-ΔN transferase activity in the dWPS only increased once GTFB-ΔN hydrolytic activity had created sufficient DP < 6 oligomers. Enzymatic fingerprinting of dATPS after GTFB-ΔN incubation revealed that malto-oligomers DP > 6 will not act as acceptor at all. This indicates that all glucans in the final product are either α-(1 → 6) linked glucans linked to α-(1 → 4) linked oligomer acceptors ranging from DP 1 to DP 5, as seen in Fig. 6, small α-(1 → 4) linked left-over acceptors ranging from DP 1 to DP 5 or purely α-(1 → 6) linked oligomers. The addition of small di- or oligomer acceptors could potentially increase the rate and efficiency of GTFB-ΔN transferase activity leading to less hydrolysis and higher level of α-(1 → 6) glycosidic linkages. Altogether, we can conclude that the presence of α-(1 → 4) linked oligomer acceptors (DP < 6) plays a decisive role for the rate and level of formation of α-(1 → 6) glycosidic linkages during IMMP synthesis.

4. Conclusions

In this study we found that the GTFB-ΔN elongation of α-(1 → 6) linked glucans goes much further than reported previously. GTFB-ΔN is able to synthesize IMMPs having a larger molecular weight than the initial α-(1 → 4) linked substrates. Enzymatic fingerprinting revealed that GTFB-ΔN is able to synthesize α-(1 → 6) linked glucans even up to 21 kDa (DP 130). In addition, enzymatic fingerprinting revealed that GTFB-ΔN is not able to use malto-oligomers < DP 6 as a donor substrate, but only as acceptor molecule. In contrast, linear α-(1 → 4) linked glucans of DP > 6 can only be used as a donor substrate by GTFB-ΔN, not as acceptor molecule. The use of small linear acceptor molecules during GTFB-ΔN incubation will therefore be of great interest for the targeted synthesis of IMMPs in the future.

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CRediT authorship contribution statement

Cynthia Klostermann: Investigation, Methodology, Writing – review and editing.

Piet van der Zaal: Investigation, Methodology, Writing – original draft.

Piet Buwalda: Conceptualization, Writing – review and editing, Supervision.

Henk Schols: Conceptualization, Writing – review and editing, Supervision.

Harry Bitter: Writing – review and editing, Supervision.

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