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ABSTRACT

Starch provides our main dietary caloric intake and over-consumption of starch-containing foods results in escalating life-style disease including diabetes. By increasing the content of α-1,6 branch points in starch, digestibility by human amylolytic enzymes is expected to be retarded. Aiming at generating a soluble and slowly digestible starch by increasing the content and changing the relative positioning of the branch points in the starch molecules, we treated cassava starch with amylomaltase (AM) and branching enzyme (BE). We performed a detailed molecular analysis of the products including amylopectin chain length distribution, content of α-1,6 glucosidic linkages, absolute molecular weight distribution and digestibility. Step-by-step enzyme catalysis was the most efficient treatment, and it generated branch structures even more extreme than those of glycogen. All AM- and BE-treated samples showed increased resistance to degradation by porcine pancreatic α-amylase and glucoamylase as compared to cassava starch. The amylolytic products showed chain lengths and branching patterns similar to the products obtained from glycogen. Our data demonstrate that combinatorial enzyme catalysis provides a strategy to generate potential novel soluble α-glucan ingredients with low dietary digestibility assets.

KEYWORDS: Amylomaltase, Branching enzyme, Clean modification, Branched glucan, Slow carbohydrate
1. INTRODUCTION

Starch is the major reserve carbohydrate of higher plants, especially in tubers, roots and grains. Starch normally consists of two discrete molecular fractions. Amylose makes up 14–27% of the starch depending on the starch source. This polysaccharide is typically 100–10,000 glucosyl units large, it has an α-1,4 backbone structure, and it is only slightly branched by α-1,6 linkages. Amylopectin makes up 73-86% depending on the starch source, being more than 100-fold larger than amylose, and contains approximately 5% clustered α-1,6 linkages (Dziedzic & Kearsley, 1995; Damager, Engelsen, Blennow, Møller & Motawia, 2010). Cassava is one of the most widely distributed crops on earth and it is cultivated crops in numerous tropical countries. Cassava starch is easily extractable and very pure products are obtained due to the very low protein and lipid content of cassava. The starch plays an important industrial role, both in native and modified forms, which are widely used in the food and non-food industries.

For starch digestion in humans, α-amylase first hydrolyzes starch to produce α-limit dextrins, followed by complete hydrolysis to glucose by the mucosal α-glucosidases in the small intestine. The rate of starch digestion is controlled by several factors such as its physicochemical properties, the structural architecture of the starch-containing food matrix and the presence of other dietary components (Butterworth et al., 2012). It is known that α-1,6 linkages in starch are hydrolysed at a lower rate than are α-1,4 linkages (Lee et al., 2013). Generally, the initial reaction rate of this hydrolysis decreases with increasing degree of polysaccharide branching. This effect is mainly due to steric hindrance in the active site of the hydrolase exerted by α-1,6 bonds (Park & Rollings, 1994). Therefore, the production of modified starch having low digestion rate has drawn interest because this starch is considered to offer an advantage by inducing only a slow increase of the postprandial blood glucose levels, thereby sustaining the
blood glucose levels over time (Lehmann & Robin, 2007).

Enzymatic methods are now emerging as alternative clean technologies to provide more environment and consumer safe solutions for starch modification. In contrast to physical and chemical methods, which often produce unpredicted by-products, enzyme-assisted catalysis can be more specifically controlled and it operates under very mild conditions, thus reducing the risk for producing harmful or unwanted by-products (Butler, Van der Maarel & Steeneken, 2004).

Glucanotransferases belong to specific enzyme families and they catalyse transfer segments of α-glucans in distinct ways. As compared to hydrolases, the use of glucanotransferases has the advantage of retaining higher product yield of high-molecular products. Amylomaltase (AM; (1→4)-α-D-glucan:(1→4)-α-D-glucan 4-α-D-glycosyltransferase; E.C. 2.4.1.25. belonging to either glucosyl hydrolase family 77, GH77, www.CaZy.org) is an intracellular enzyme that cleaves α-1,4 glucosidic linkages followed by a condensation of a new α-1,4 linkages within the same α-glucan molecule (intra-molecular) or between the different molecules (intermolecular) (Boos & Shuman, 1998). Due to its transfer of identical linkages, this catalytic reaction is also termed disproportionation. Furthermore, AM can catalyze intra-molecular α-glucan transfer reactions to create cyclic molecules (cyclization) and also has minor hydrolytic activity. As an effect, coupling reactions can occur leading to “reverse cyclization”, in which cycloamylose is opened by the enzyme and transferred to an acceptor as a linearized fragment (Fujii et al., 2007). However, the unique action modes of different AMs depend on the species of microorganisms.

Branching enzyme (BE, (1→4)-α-D-glucan:(1→4)-α-D-glucan 6-α-D-[(1→4)-α-D-glucano]-transferase, EC 2.4.1.18, glucosyl hydrolase family 13 or 70, GH13, GH70, www.CaZy.org) acts on α-1,4 glucosidic linkages to produce a branched α-glucan by intra- or
inter-molecular α-1,6 glucosidic transfer (Okada, Kitahana, Yoshikawa, Sugimoto & Sugimoto, 1984). Moreover, it has been demonstrated that BE also catalyzes the cyclization of amylase and amylopectin (Takata, Takaha, Okada, Hizukuri, Takagi & Imanaka, 1996). Due to the high branching and relatively low molecular size, the products are highly soluble in water, as compared to normal starch, giving a highly stable clear solution and reduced retrogradation (Takata, Takaha, Okada, Hizukuri, Takagi & Imanaka, 1996). Very recently, BE was demonstrated to possess a minor transferring activity of α-1,4 linkages to create new α-1,4 linkages in analogy to AM, yielding elongated linear chains (Roussel et al., 2013).

In this study, we seek to generate more compact branching and homogeneous size distribution of the product by employing combinations of AM and BE in sequence (AM→BE, BE→AM→BE) or simultaneously (AM&BE) expecting to produce a range of differently, highly branched glucan structures with increased resistance towards important dietary amylases. Our data demonstrate that combinatorial glucanotransferase catalysis provides a clean strategy to generate potential novel soluble α-glucan ingredients with low dietary digestibility assets. The strategy is expected to be applicable also for other combinatorial transferase systems.

2. MATERIALS AND METHODS

2.1 Materials

Cassava starch was obtained from SanguanWongse Industries Co., Ltd. (NakhonRatchasima, Thailand). Preparations of BE (Viksø-Nielsen, Blennow, Nielsen & Møller, 1998) and AM were kindly provided from Novozymes (Bagsvaerd, Denmark). Isoamylase (EC 3.2.1.68, specific activity 210 U/mL) was obtained from Megazyme (Wicklow, Ireland). Porcine pancreatic α-amylase (PPA, EC 3.2.1.1, specific activity 22 U/mg), glucoamylase (GA, EC
3.2.1.3, specific activity 129 U/mg) from *Aspergillus niger*, PGO (peroxidase and glucose oxidase) enzyme kit for glucose determination, potato soluble starch and glycogen type VII from mussel (*Mytilus edulis*) were purchased from Sigma-Aldrich (Missouri, USA). Enzyme activity units of isoamylase, PPA and GA are given according to the supplier.

### 2.2 Enzymatic modification

#### 2.2.1 Cassava starch treated with AM followed by BE (AM→BE)

The AM-treated starch was produced mainly according to van der Maarel (van der Maarel, Capron, Euverink, Bos, Kaper & Binnema, 2005) procedure with specific modifications. Cassava starch was suspended in MilliQ water (10% (w/v)) adjusted to pH 6.0 with 50 mM phosphate buffer. The suspension was heated to 75 °C in a water bath for 15 min and then autoclaved at 121 °C for 15 min. AM (10 U/g starch) was added to the gelatinized cassava starch paste and incubated at 70 °C for 3h or 24h and then terminated by boiling at 100 °C for 30 min. The pH was adjusted to 6.5 using 50 mM phosphate buffer and BE (4,000 U/g starch) was added and the reaction mixture incubated at 60 °C for 24h. The reaction was terminated by heating in boiling water bath for 30 min, trace insolubles removed by centrifugation (1,500xg for 20 min) and the α-glucan product was recovered and dried at 50 °C overnight.

#### 2.2.2 Cassava starch treated with BE, AM and BE in sequence (BE→AM→BE)

A gelatinized cassava starch paste was prepared as mentioned above, pH adjusted to 6.5 with 50 mM phosphate buffer, BE (4,000 U/g starch) was added and the mixture incubated at 60 °C for 24h. After termination of the reaction at 100 °C for 30 min, AM (10 U/g starch) was added and incubated at pH 6.0, 70 °C for 3h or 24h. The reaction was terminated by boiling at 100 °C for 30 min. In the last step, BE was added and incubation was performed under optimal condition
for each enzyme as described above. The obtained product was then handled for storage as
described above.

2.2.3 Cassava starch simultaneously treated with AM and BE (AM&BE)

The pH of gelatinized cassava starch paste was adjusted to 6.5 with 50 mM phosphate
buffer, AM (10 U/g starch) and BE (4,000 U/g starch) were added and the mixture incubated at
60 °C for 3h or 24h. Another set of gelatinized cassava starch was prepared, pH adjusted to 6.0
with 50 mM phosphate buffer, AM (10 U/g starch) and BE (4,000 U/g starch) were added and
the mixture incubated at 70 °C for 3h or 24h. The product was collected and dried as above.

2.3 Iodine complexation

Iodine colorimetric analysis was carried out mainly as described by Wickramasinghe et
al. 2009. Cassava starch, glycogen, potato soluble starch or enzyme-modified starches (20 µg
each) were suspended in 1 mL of 1 M NaOH with shaking (1,200 RPM in a Thermomixer,
Eppendorf, Germany) overnight until completely dissolved. 50 µL sample was added to 20 µL of
the diluted iodine solution (0.26 g I₂ and 2.6 g KI in 10 mL water, diluted 60 times in 100 mM
HCl) in a microtiter plate well, and absorbance was recorded (Spectramax M5, Molecular
Devices, Sunnyvale, CA, USA) from 350 to 750 nm every 5 nm and the wavelength at maximum
absorbance (λ-max) was identified for each scan (Wickramasinghe, Blennow & Noda, 2009).

2.4 β-amylolysis limit

The analysis procedure was slightly modified from that of Hood and Mercier (1978). The
α-glucan solution (1.5 mL, 0.5% w/v in 90% DMSO) was mixed with an acetate buffer solution
pH 4.8 (0.3 mL, 0.2 M), β-amylase solution (0.2 mL, 20 units/mL) and deionized water (1.0 mL)
were added and mixed, and the solution was incubated at 37 °C for 48 h. The reducing sugar content and total sugar content were measured. The percentage of β-amylolysis limit was calculated using a following equation:

\[
\beta\text{-amylolysis limit (\%) } = \left( \frac{\text{Reducing sugar after hydrolysed} - \text{Reducing sugar of blank}}{\text{Total sugar after hydrolysed} - \text{Total sugar of blank}} \right) \times 100
\]

2.5 Chain-length distribution of debranched α-glucan by high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

Enzyme-modified starches were gelatinized by boiling and enzymatically debranched by using 2.4 U of isoamylase per 5 mg of sample at 40 °C. The obtained linear α-glucan fragments were analyzed by high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD, Dionex, Sunnyvale, CA, USA). Samples of 20 µL (100 µg of linear α-glucan) were injected on a CarboPac PA-200 column using 0.4 mL/min flow rate, 150 mM isocratic NaOH and the following NaOAc gradient profile: 0–5 min: 0–110 mM linear gradient, 5–130 min: 110–350 mM convex gradient. Single peaks were integrated and, corrected for the detector response (Viksø-Nielsen, Blennow, Nielsen & Møller, 1998).

2.6 Molecular weight distribution, polydispersity index and intrinsic viscosity analysis by size-exclusion chromatography with triple detection array (SEC-TDA)

The molecular weight distribution (Mw), the polydispersion index (Mw/Mn) and the hydrodynamic volume (Rh) were determined by size exclusion chromatography (SEC) using a Viscotek System (Malvern, UK) equipped with a GS-520 HQ column (Shodex) attached to a TDA302 module (Triple detector array) consisting in a refractive index detector (RI), a four-bridge visco-meter detector (VIS) and a light scattering detector (LS). The LS consisted of a
right angle light scattering (RALS) and a low angle light scattering (LALS) that measure the scattered light 7° and 90° with respect to the incident beam. The calibration of the instrument was made using pullulan (50 kDa, polydispersion 1.07, Showa Denko) as a standard, solubilized in MilliQ water (1 mg/mL) and mechanically shaken at 99 °C for 120 min at 1,000 rpm. Isocratic elution was made using 50 mM ammonium formate (HCO₂NH₄) buffer, with a flow of 0.5 mL/min. Samples were filtered through a 0.22 µm syringe filter and automatically injected (GPC max module) into the column. The injection volume was 50 µL, the column temperature 60 °C. The analysis was performed using the OmniSec Software 4.7 (Malvern Instrument, ltd).

2.7 α-1,6 glucosidic linkages determination by nuclear magnetic resonance (NMR)

The α-glucan samples were dissolved in 500 µL D₂O (Cambridge Isotope Laboratories, Andover, MA, USA) to concentrations of 0.3% (w/v) under gentle heating. ¹H-NMR spectra were recorded on a Bruker (Fällanden, Switzerland) DRX spectrometer equipped with a TCI CryoProbe and an 18.7 T magnet (Oxford Magnet Technology, Oxford, UK) at 37 ºC. Spectra were recorded by sampling 16,384 complex data points during an acquisition time of 1.7 sec, employing 32 transients and a recycle delay of 10 sec for reliable quantifications. NMR spectra were processed using Bruker Topspin 2.1 software with zero filling in all dimensions and mild resolution enhancement. For the latter, we employed a time domain Lorentzian-Gaussian Transformation with a line broadening of -1 Hz and a Gaussian broadening of 0.3. Anomeric signals in branch point α-1,6 linkages were quantified relative to anomeric signals in α-1,4 linkages.
2.8 Cycloglucan content and confirmation by matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS)

Cycloglucan content was expressed in terms of % of GA-resistant molecules. The GA-resistant molecules content was determined following Takaha et al., (1996). The enzyme-modified starch products were dissolved in 50 mM acetate buffer pH 5.5 for 1 h at 37 °C and incubated with 10 U of GA only or with 10 U of GA and 10 U of PPA. The reaction was terminated by boiling the mixture for 5 min and the glucose released from the modified-samples was measured. The content of GA-resistant molecules was calculated by subtracting the content of glucose released by GA-PPA from that of GA.

The enzyme-modified starch products were dissolved in DMSO by vortexing and diluted with water to a final concentration of 1 µg/mL. A solution of 2,5-dihydroxybenzoic acid in 30% acetonitrile in water with 0.1% TFA (20 mg/mL) was prepared as the matrix. The matrix solution (2 µL) was combined with the diluted analyte solution (2 µL) and this solution (0.5 µL) was added to the target and air-dried. MALDI-TOF MS was performed using a Bruker Daltonics Microflex instrument operating in reflectron mode. A 340 nm laser was used and mass spectra were typically accumulated from 1,000 laser shots. Spectra were generally acquired over a 4,000 m/z range (from 500 Da to 4,500 Da, with matrix suppression up to 400 Da).

2.9 In vitro starch digestion analysis

In vitro starch degradation was analyzed by a modification of the Englyst method using gelatinized cassava starch, potato soluble starch and glycogen type IV (all gelatinized at 100°C for 30 min) and modified glucan products. Starch and glucan samples (2% (w/v) in 250 µL), were incubated in triplicates with 20 U of each PPA (Sigma A3176) and GA (Sigma 10113) in
20 mM sodium phosphate buffer with 6.7 mM sodium chloride (pH 6.0) at 37 °C for 0, 10, 30, 60, 120, 240, 360, 480, 1440 min. Enzyme reaction was terminated by adding 30 µL 100 mM HCl. The rate of starch digestion was expressed as % of glucose released from the total starch or α-glucan over the time period (Englyst, Kingman & Cummings, 1992) by using PGO enzymes (Sigma P7119).

The glucose released (%) was calculated using the following equation:

\[
\text{Glucose released (\%)} = \left( \frac{\text{Total weight of glucose} \times 0.9}{\text{Weight of enzyme-modified starches}} \right) \times 100
\]

where 0.9 is the molar mass conversion from glucose to anyhydroglucose.

3. RESULTS

3.1 Structural analysis of AM and BE modified products

Cassava starch was treated with AM and/or BE either separately, in sequential steps, or simultaneously for different time periods as indicated in Figure 1 and Table 1. The \( \lambda_{\text{max}} \) of the iodine-\( \alpha \)-glucan complexes for all samples were suppressed to values close to the \( \lambda_{\text{max}} \) of glycogen (Table 1). In many cases, the \( \lambda_{\text{max}} \) was lower than 440 nm demonstrating the formation of very densely branched products. The \( \alpha \)-1,6 linkage content determined by \( ^1 \)H-NMR is given in Table 1 and relevant signals are highlighted in the NMR spectra of Supplementary Figure 1. The AM\( \rightarrow \)BE and BE\( \rightarrow \)AM\( \rightarrow \)BE modified starches had higher content of \( \alpha \)-1,6 linkages compared to cassava starch and glycogen. Interestingly, the BE\( \rightarrow \)AM\( \rightarrow \)BE product showed the highest content of \( \alpha \)-1,6 glucosidic linkages as compared to control BE\( \rightarrow \)X\( \rightarrow \)BE (Table 1, X denoting no intermittent treatment) demonstrating an important role of AM to provide efficient substrate for BE to further increase the degree of branching. The five samples with the highest content of
α-1,6 linkages were selected for further molecular analysis: AM (3h, 24h) → BE, BE → AM (3h, 24h) → BE, and AM&BE (60°C, pH 6.5, 24h).

**Table 1.** Maximum wavelength absorption of iodine-α-glucan complex and content of α-1,6 glycosidic linkages (%) of the control and enzyme-modified starches. X denotes no enzyme treatment in that step. nd: not determined, BE: BE-treated starch for 24 h

<table>
<thead>
<tr>
<th>Samples</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt; (nm)</th>
<th>α-(1,6) linkages (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cassava starch</td>
<td>565</td>
<td>4.9</td>
</tr>
<tr>
<td>Glycogen</td>
<td>440</td>
<td>9.7</td>
</tr>
<tr>
<td>Potato amylose</td>
<td>630</td>
<td>nd</td>
</tr>
<tr>
<td>Potato amylopectin</td>
<td>540</td>
<td>nd</td>
</tr>
<tr>
<td>BE</td>
<td>525</td>
<td>7.8</td>
</tr>
<tr>
<td>AM 3h → BE</td>
<td>500</td>
<td>9.9</td>
</tr>
<tr>
<td>AM 24h → BE</td>
<td>470</td>
<td>10.0</td>
</tr>
<tr>
<td>BE → X → BE</td>
<td>415</td>
<td>8.3</td>
</tr>
<tr>
<td>BE → AM 3h → BE</td>
<td>420</td>
<td>13.1</td>
</tr>
<tr>
<td>BE → AM 24h → BE</td>
<td>410</td>
<td>10.9</td>
</tr>
<tr>
<td>AM&amp;BE 60°C, pH 6.5, 3h</td>
<td>560</td>
<td>5.1</td>
</tr>
<tr>
<td>AM&amp;BE 60°C, pH 6.5, 24h</td>
<td>425</td>
<td>8.7</td>
</tr>
<tr>
<td>AM&amp;BE 70°C, pH 6.0, 3h</td>
<td>560</td>
<td>4.7</td>
</tr>
<tr>
<td>AM&amp;BE 70°C, pH 6.0, 24h</td>
<td>540</td>
<td>6.4</td>
</tr>
</tbody>
</table>

### 3.2 Chain length distribution

#### 3.2.1 Single catalysis

The effects of AM and BE catalysis on the average chain-length distribution or degree of polymerization (DP) of modified cassava starch were investigated by using HPAEC-PAD. Cassava starch only treated with BE for 24h showed a reduced number of chains with DP ≥ 20 whilst the proportion of chains shorter than DP 20 were increased as compared to cassava starch.
(Figure 1B). When cassava starch was treated with AM, the chain length profile shows a specific increase in DP $\leq 7$ and DP $\geq 25$ while DP 7-25 were decreased (Figure 1C). The appearance of the low DP chains are supposedly originating from residual segment from the donor chains after transglycosylation and from minor hydrolase activity of BE and AM. The hydrolytic activity of AM is considered minor (Kaper et al., 2007; Kaper, van der Maarel, Euverink & Dijkhuizen, 2004; van der Maarel, Capron, Euverink, Bos, Kaper & Binnema, 2005) and was in our preparations estimated to 0.73 % and 0.13 % of the transferase activities for BE and for AM, respectively as determined by the Ceralpha method (Megazyme, Wicklow, Ireland).

3.2.2 AM→BE sequential catalysis

In an attempt to change the relative positions to further increase the number of branch points in the $\alpha$-glucan product, the cassava starch was subjected to AM treatment for 3h or 24h preceding BE treatment (AM→BE). Such modification is expected to re-distribute the chain segments in relation to the $\alpha$-1,6 linkages providing a different substrate for the following BE catalysis. All AM→BE treated samples showed a substantial amount of chains smaller than DP 25 that were accumulated at the expense of chains longer than DP 25 (Figure 1E). These results suggest that BE prefers to use the AM elongated chains of DP $\geq 25$ in the amylopectin (Figure 1C). The effect of the incubation time with AM was minor as supported by the minor difference found between the AM 3h→BE and AM 24h→BE samples (Figure 1C).

3.2.3 BE→AM→BE sequential catalysis

In order to further investigate the possibility to further increase branching of the $\alpha$-glucans, samples were sequentially treated with BE for 24h, AM for 3 or 24h and last with BE for 24h (BE→AM→BE, Figure 1F). This approach provides new potential $\alpha$-glucan substrates
for BE in the last step, potentially further increasing the branching. In order to investigate the
efficiency of isoamylase debranching, to identify possible closely positioned α-1,6 branch points
not accessible for isoamylase catalysis, β-amylolysis after isoamylase debranching was
performed (Supplementary Table 1). The β-amylolysis for the BE→AM→BE modified starches
spanned 92.8-93.0% as compared to the control glycogen (98.4%) and BE treated starch (99.4%)
demonstrating that isoamylase could not completely debranch these highly branched products,
supposedly due to closely positioned α-1,6 branch points. For the sample treated with BE→AM
3h→BE, a high content of very short α-glucans chains ranging DP 3-12 were formed (Figure 1F) and in the raw chromatograms minor peaks were identified as branched α-glucans.
Compared to the BE→AM 24h→BE sample, BE→AM 3h→BE showed lower accumulation
of chains ranging from DP 3-15. Furthermore, AM very inefficiently acted on BE treated (highly
branched) substrate (Figure 1D); hence, very subtle differences in substrate structure in the
region DP 8-15 determine the substrate recognition requirements for the BE to act upon to
generate substantial differences in BE chain transfer rates seen in Figure 1F. Notably, the
BE→X→BE sample did not gain high content of α-1,6 branch points despite the extensive BE
treatment for two consecutive time periods supporting that AM catalyses the disproportionation
and/or cyclization of amylopectin (Hansen, Blennow, Pedersen, Norgaard & Engelsen, 2008),
increasing the catalytic rate of BE.

3.2.4 Simultaneous AM&BE catalysis

Finally, we investigated the simultaneous synergistic effects of AM and BE by adding
AM and BE simultaneously (AM&BE) in the system using the optimal catalytic conditions for
each enzyme respectively, incubating for 3h or 24h (Figure 1G). The optimum conditions for BE
from *Rhodothermus obamensis* activity is 60 °C at pH 6.5 while 70 °C at pH 6.0 is the optimum
condition for AM from *Thermus thermophilus*. Incubation at the optimum condition of BE for 24h increased the portion of smaller α-glucan chains (DP 3-20) as compared to the AM optimum condition. For this sample, longer chains (DP > 20) were consumed by chain transfer while for the other three incubation conditions (60 °C for 3h or 70 °C for 3h or 24h, respectively) the α-glucan chains of approximately DP 25-40 were increased. As expected, longer incubation time at optimum condition for BE resulted in a highly branched product (Table 1). Likewise, after incubation for shorter time at the optimum condition for BE, similar distribution was found as for the optimum condition for AM. Nevertheless, AM and BE still catalyzed chain transfer although the conditions were not optimal, confirming that both amylose and amylopectin are substrates for the AM and BE in the mixed systems, but it should be noted that both enzymes has relatively higher affinity for amylose than for amylopectin (Shinohara, Ihara, Abo, Hashida, Takagi & Beck, 2001). Most importantly, simultaneous catalysis of AM and BE did not result in the very highly branched product as demonstrated for the sequential action (Table 1), suggesting that products generated as intermediates in the AM&BE combined reaction mixture were non-optimal substrates for further branching.
Figure 1. Chain length distribution analysis and difference plots of debranched α-glucan samples demonstrating differences due to additional sequential enzyme catalysis with reference to the preceding enzyme step. (A) Chain length distributions of enzyme treated samples compared to cassava starch and glycogen. (B-G) Difference plots relative to controls as indicated. (B) BE treated samples as compared to cassava starch. (C) AM treated samples. (D) AM treated sample as compared to BE treated cassava starch. (E) BE treated sample as compared to AM treated cassava starches. (F) BE treated sample as compared to BE→AM treated cassava starch using BE treated cassava starch as control. (G) Simultaneous action of AM&BE using cassava starch as control. The Y-axis in A denotes the peak areas standardized to 100%.

3.3 Molecular size and cyclo-structures

Intra-chain transfer leads to cyclisation and a reduction in the molecular size. In order to test if any of the enzyme treatments resulted in amylopectin cluster and/or cyclo-amylopectin formation, average molecular weight was analysed. All AM and BE-treated samples showed decreased average molecular weight except for the AM-treated cassava starch (Figure 2). As compared to the glycogen elution profiles, the average molecular weights of the samples were lower, indicating hydrolysis of α-1,4 linkages between amylopectin clusters as mentioned in section 3.2.1 together with the formation of amylopectin cyclo-clusters during the branching reaction. The reduction of molecular size following BE treatment has been reported elsewhere (Kim, Ryu, Bae, Huong & Lee, 2008; Le et al., 2009) and this is the first evidence provided for cyclo-amylopectin cluster formation catalyzed by the *Rhodothermus obamensis* BE. For AM catalysis, cyclization of amylopectin to form amylopectin clusters has been demonstrated (Hansen, Blennow, Pedersen, Norgaard & Engelsen, 2008). Hydrolytic activities in the AM and
BE used in this study were low; 0.73 % and 0.13 % for BE and for AM, respectively as mentioned above. Hence the reduction of molecular size following AM and/or BE catalysis is likely a combined effect of minor hydrolysis and the formation of amylopectin cluster and cyclo-amylopectin clusters. The samples were analysed by MALDI-TOF (Supplementary Figure 2). Interestingly, the smallest cyclic α-glucan found was γ-cyclodextrin (DP 8, m/z 1320) and this compound was detected in all modified starches and especially in the AM treated samples. The cycloglucan content, as analysed in terms of GA-resistant molecules, was very low (0.1-1.8%). Polydispersity index (Mw/Mn) and hydrodynamic volume (Rh) (Supplementary Table 2) showed that the modified starch samples had (Mw/Mn) values as for glycogen and the product obtained after sequential enzyme catalysis shows decreased Rh corresponding to the reduction of the average molecular weight.
Figure 2. Size distributions of cassava starch, glycogen and selected enzyme-modified cassava starch products as analyzed by size-exclusion chromatography with triple detection array (SEC-TDA).

3.4 Amylolytic susceptibility evaluated in vitro

To evaluate the susceptibility to amylolytic digestion of the AM and BE treated starches, six selected samples were subjected to combined PPA and GA treatment. Generally, the modified products showed less susceptibility to PPA and GA digestion than gelatinised cassava starch. The digestion profiles of the five selected samples compared to cassava starch and glycogen (Figure 3) were fitted to a first order kinetic model, $C = 1-e^{kt}$, where t is the digestion
time (min), C is the fraction of digested starch at specified reaction time, and k is the digestion rate constant (min$^{-1}$). The value of k was obtained from the slope of a linear-least-squares fit of a plot of ln(1 −C) against t (Butterworth, Warren, Grassby, Patel & Ellis, 2012; Zhang, Dhital & Gidley, 2013) (Table 2 and Figure 3). The maximum degradation was found to be lower for all the enzyme-modified samples than for the cassava starch demonstrating the presence of resistant α-glucans. Specifically, the initial hydrolysis rates for the enzyme-modified starches were slower than for the cassava starch control. However, a minor curvature in the first order plots of all samples (Supplementary Figure 3) suggests that there were two stages of kinetic process with different rates. Therefore, the k-values obtained from a first order kinetic model derive from two stages of the digestion. The k-values of the modified starches were significantly lower than for cassava starch control supporting a previous study on the modification of starches with BE combination with β-amylase (Le et al., 2009; Lee et al., 2007). A high k-value indicates high susceptibility of PPA and GA catalysis. The k-value for cassava starch and glycogen were 5.7x10$^{-3}$ min$^{-1}$ and 1.7x10$^{-3}$ min$^{-1}$, respectively. The corresponding values for the BE$\rightarrow$AM 3h$\rightarrow$BE and BE$\rightarrow$AM 24h$\rightarrow$BE products were 1.4x10$^{-3}$ min$^{-1}$ and 1.6x10$^{-3}$ min$^{-1}$, respectively and for the AM 3h$\rightarrow$BE and AM 24h$\rightarrow$BE were 1.9x10$^{-3}$ min$^{-1}$ and 2.1x10$^{-3}$ min$^{-1}$, respectively. Hence, the BE$\rightarrow$AM$\rightarrow$BE products had significantly lower k-values compared with the others analysed samples except for glycogen. The difference was more pronounced at maximum digestion reflecting a pronounced effect at the final digestion level by the PPA and GA (Figure 3). The levels of glucose released from all samples spanned 64.7-87.7%. The remaining structures can be considered as very resistant towards amylolysis.

**Table 2.** Digestion rate coefficient (k, min$^{-1}$) of cassava starch, glycogen and enzyme-modified starches. BE: Starch treated with BE for 24 h. The data are averages of two
measurements ± SD. Means in columns with different letters are significantly different (p<0.05) by general linear model.

<table>
<thead>
<tr>
<th>Samples</th>
<th>k (min⁻¹) x10⁻³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cassava starch</td>
<td>5.7±0.2ᵃ</td>
</tr>
<tr>
<td>Glycogen</td>
<td>1.7±0.2ᵇᶜ</td>
</tr>
<tr>
<td>BE 24h</td>
<td>2.0±0.3ᵇ</td>
</tr>
<tr>
<td>AM 3h → BE</td>
<td>1.9±0.1ᵇᶜ</td>
</tr>
<tr>
<td>AM 24h → BE</td>
<td>2.1±0.2ᵇ</td>
</tr>
<tr>
<td>BE → AM 3h → BE</td>
<td>1.4±0.9ᶜ</td>
</tr>
<tr>
<td>BE → AM 24h → BE</td>
<td>1.6±0.2ᶜ</td>
</tr>
<tr>
<td>AM&amp;BE 60°C pH6.5 24h</td>
<td>2.1±0.1ᵇ</td>
</tr>
</tbody>
</table>
Figure 3. Time course digestion profiles of enzyme-modified samples digested with PPA and GA compared with cassava starch.
The resistant malto-oligosaccharides obtained after 24h hydrolysis with PPA and GA were analysed using HPAEC-PAD (Figure 4). The chromatographic fingerprint profiles of the products demonstrate high amounts of glucose and maltose. The resistant malto-oligosaccharides included a range of DP $\geq 4$. All modified starches and glycogen showed higher content of resistant malto-oligosaccharides as compared to cassava starch. The BE$\rightarrow$AM$\rightarrow$BE modified starches were initially relatively slowly hydrolyzed by PPA and GA and finally reached lower levels of hydrolysis as compared to the other products. As a conclusion, the difference in the hydrolytic profiles is supposedly directly linked to the presence of densely branched products, especially in the BE$\rightarrow$AM 3h$\rightarrow$BE treated starches.
**Figure 4.** Oligosaccharide profiles of enzyme-modified starches by PPA and GA after *in vitro* digestion as analysed by Dionex chromatography. Top chromatogram shows linear standards. Malto-oligosaccharides eluting at intermediate positions indicate the presence of differently branched molecules that are partly resistant towards amylolytic degradation.

**4. DISCUSSION**

**4.1 Synergistic AM and BE catalysis on cassava starch structure**

In this study, combinatorial BE and AM catalysis for starch modification was introduced as a new strategy to achieve extensive branching of a starch substrate, thereby suppressing amylolytic susceptibility. The small branch chains fraction of BE starches modified for 24h were increased comparable to that of glycogen (Figure 1B). Similar structures were reported for BE from *Bacillus subtilis* and *Rhodothermus obamensis* (Kim, Ryu, Bae, Huong & Lee, 2008; Le et al., 2009) demonstrating that BE generally and specifically catalyzes the formation of extensive α-1,6 linkages. It should be noted that BE, as a minor activity, is also capable of re-distributing α-1,4 linkages in a minor transglycosidation reaction in a similar way as AM (Roussel et al., 2013). The presence of low molecular weight α-glucans (Figure 2) in the enzyme-modified starch products indicated a hydrolytic and/or disproportionation activity of AM and BE (Figure 1C) that agrees with previous data (Hansen, Blennow, Pedersen & Engelsen, 2009). The disproportionation activity of AM demonstrates that AM can provide longer α-glucan chains for further efficient chain transfer catalyzed by BE. The dense branching of the product from the AM→BE treatment (Figure 1E) showed that BE can use the outer chains of the AM treated products to create new branch points (Table 1).
A substantial collected volume of evidence points at health-associated assets of glycogen-like structures and their potential in functional foods (Kajiura, Kakutani, Akiyama, Takata & Kuriki, 2008). Glycogen has a degree of branching that is about twice that of amylopectin (Kajiura et al., 2011) and the combined AM and BE catalysis approach in one case surpassed the chain length distribution of glycogen. This finding highlights the value of combining different enzyme activities to generate products more extreme than found in nature. The BE→AM→BE treated starch generated the highest content of α-1,6 linkages with a chain-length distribution very different to that of cassava starch (Table 1). A plausible explanation for this effect could be that the BE→AM-catalysed sequential reaction, provides optimum chain length and differently positioned chains for BE to create further branch points in the last BE catalyzed step. However, previous data suggest that AM preferably catalyses the transfer of longer chains like those found in amylose (Bhuiyan, Kitaoka & hayashi, 2003). Therefore, for the BE→AM catalysed step, the restriction of such long amylose chains in the BE treated sample would force AM to act on sub-optimal, short chains and amylopectin clusters. This effect was supported by the minor difference found in the sequential BE→AM (Figure 1D) as compared to the AM treated sample chain profile (Figure 1C). AM is thus potentially mainly involved in transfer of larger fragments e.g. rearrangement of amylopectin clusters and transfer of amylose segments (Hansen, Blennow, Pedersen & Engelsen, 2009). However, rearrangements of amylopectin clusters can enhance BE catalysis in the last step by providing steric access to BE. By using the combined BE→AM→BE protocol, the chain length density of glycogen was surpassed generating a very densely branched α-glucan products.

We observed a distinct difference between simultaneous and sequential catalysis of AM&BE. The simultaneous action of AM&BE increased the branch points in the products as
compared to control but the effect was less pronounced than for the sequential AM→BE and BE→AM→BE treatments. These data suggest a catalytic competition between AM and BE on long chains during catalysis. At optimal conditions for AM (70 °C, pH 6.0), BE activity was sub-optimal and vice versa. High BE activity efficiently removes amylose from the system preventing AM to transfer amylose segments to amylopectin or rearrange amylopectin cluster (Bhuiyan, Kitaoka & Hayashi, 2003; Fujii et al., 2007). This effect potentially leads to lower content of α-1,6 linkages in the simultaneous AM&BE samples (Table 1).

Intra-molecular chain transfer may lead to the generation branched (by BE) or non-branched (by AM) cyclic structures (Bhuiyan, Kitaoka & Hayashi, 2003; Park et al., 2007; Takata, Takaha, Okada, Takagi & Imanaka, 1996; Viksø-Nielsen, Blennow, Nielsen & Møller, 1998). The smallest cycloglucan found was the eight glucose-ring γ-cyclodextrin. This is in contrast to earlier findings (Bhuiyan, Kitaoka & Hayashi, 2003; Park et al., 2007) where no γ-cyclodextrin was detected but the smallest cycloglucan found, following *Aquifex aeolicus* AM treated amylose, was DP16 (Bhuiyan, Kitaoka & Hayashi, 2003). Amylose treated with AM from *Thermus aquaticus* generated cycloamyloses down to DP19 (Park et al., 2007). We speculate that this discrepancy can be an effect of the mixed amylose and amylopectin system used in our study providing different types of substrate for the AM potentially with different helical folds.

4.2 Synergistic AM and BE Catalysis on Amylolitic Enzyme Digestion

Hypothetically, when polysaccharide branching is increased, the subsequent hydrolytic digestion rate should be suppressed as an effect of steric hindrance imparted by the increased and re-positioned α-1,6 linkages as compared to normal starch. As an effect of restrictions in PPA and GA hydrolase recognition of α-1,6 linkages, more densely branched substrates are expected
to slow down, or virtually prevent, their enzyme activity. Consistent with this hypothesis, all 
enzyme modified products showed restricted glucose release following hydrolysis. The 
BE→AM→BE products were slightly (but each one not significantly) less susceptible than 
glycogen. All enzyme-modified starches showed slow initial rates of glucose release, indicating 
that the amylopectin clusters were rearranged. Presumably, more random positions as compared 
to normal starch and/or highly branched amylopectin clusters and highly branched cyclic-
amylopectin clusters were synthesised.

The resistance towards amylolytic degradation is directly related to the affinity of the 
amylases towards specific linear and branched configurations. PPA needs at least four glucose 
units between two branch points to cleave this α-glucan molecules (Damager et al., 2005). GA 
hydrolyses α-1,4 glucosidic linkages at an approximately 30-fold higher rate than α-1,6 
glucosidic linkages (Pazur & Ando, 1960). This difference in rates indicates that the α-glucan 
molecules left after amylolytic digestion may therefore have at least one branch point left from 
the non-reducing end. The other branch positions must be close to each other and mostly 
composed of chains branched every two to four glucose units. The presence of such structures 
provides a mechanistic basis for the lower hydrolytic susceptibility of the branched products as 
compared to cassava starch. The presence of branched malto-oligosaccharides (Figure 4) 
confirms that α-1,6 linkages were present on the α-glucan chains every two to four glucose units 
which PPA could not digest. These are the shortest linear segments that PPA can cleave as 
mentioned above, resulting in larger branched malto-oligosaccharides with DP ≥ 4 (Figure 4). 
Hydrolytic, branched products likely formed following PPA catalyzed hydrolysis are shown in 
Supplementary Table 4.
Our results demonstrate the potential of using combinatorial enzyme modification, in this case the glucanotransferases AM and BE, to modify bulk starch polysaccharides and thereby modulate dietary digestibility and functionality. Such products have possible applications as soluble dietary fibers conferring prebiotic properties. The approach also reduces industrial waste as compared to classical chemical modification.

5. CONCLUSIONS

Diverse structures of highly branched, nearly monodisperse and soluble α-glucan products were produced by different combinations of AM and BE catalysis using cassava starch as substrate. Sequential BE à AM à BE catalysis resulted in more extensive branching as compared to all other enzyme treatment combinations and the products also exhibited higher branching than glycogen. These findings demonstrate the importance of combinatorial catalytic approaches to optimize the synergistic effects of α-glucantransferases in order to optimize molecular structures like α-glucan branching. All α-glucan products had slower amylolytic enzyme digestion rates as compared to native cassava starch making these α-glucans potentially prebiotic. The practical implications are found within new combinatorial and industrially up-scalable ways to produce slowly digestible carbohydrates.

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