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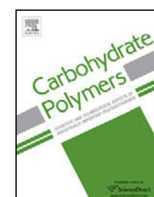

Different action patterns of glucoamylases on branched gluco-oligosaccharides from amylopectin

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ABSTRACT

A bottleneck in enzymatic starch hydrolysis, like in biofuel industry, is relatively slow degradation of branched structures compared to linear ones. This research aimed to evaluate glucoamylases for their activity towards branched gluco-oligosaccharides. The activity of seven modified glucoamylases and two homologs was compared to that of a reference glucoamylase obtained from a commercial enzyme cocktail 'Distillase® SSF'. All enzymes were evaluated for their activity towards panose (glc(α1-6)glc(α1-4)glc), pullulan and a purified branched gluco-oligosaccharide with a degree of polymerisation of 5 (bDP5) identified as glc(α1-4)[glc(α1-4)glc(α1-6)]glc(α1-4)glc. The enzymes degraded bDP5 differently, which was mainly due to variation in their capability to cleave α-(1→6)-linked or the α-(1→4)-linked glucosyl residue at the non-reducing end of the branched glucosyl residue. By comparing the enzyme activity towards bDP5 with those towards panose and pullulan, it was suggested that the activity towards bDP5 could be estimated only when the activity towards both commercial substrates was evaluated.

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1. Introduction

The role of glucoamylases is essential in starch saccharification. Glucoamylases (EC 3.2.1.3; GH Family 15, 97) are inverting enzymes that cleave one glucosyl unit each time from the non-reducing end of starch (Carbohydrate Active Enzymes database, www.cazy.org (Lombard, Golaconda Ramulu, Drula, Coutinho, & Henrissat, 2014)). Together with α-amylases, glucoamylases can degrade starch completely to glucose, because they can cleave both α-(1→4)- and α-(1→6)-linkages (Pazur & Ando, 1960). Starch is composed of amylose, which contains only (1→4)-linked α-glucosyl residues, and amylopectin, in which some of the (1→4)-linked α-glucosyl residues have an α-(1→6)-linked branch (Zobel, 1988). From amylose and amylopectin, α-amylases produce linear as well as branched gluco-oligosaccharides (Van der Maarel, Van der Veen, Uitdehaag, Leemhuis, & Dijkhuizen, 2002), which can be completely degraded by glucoamylases to glucose. However, the rate of conversion of linear versus branched oligosaccharides differs. In a previous study, it was reported that linear maltodextrins were degraded faster than branched gluco-oligosaccharides by a glucoamylase from *Hypocrea jecorina*, because the glucoamylase cleaved the α-(1→4)-linkage close to the branching point more slowly than those further away from the branching glucose (Jonathan, Van Brussel, Scheffers, & Kabel, 2015). Hence, glucoamylase activity on branched structures is one of the limiting factors for complete saccharification of starch.

An improved variant of glucoamylase with a higher affinity for branched structures could potentially result in an improved saccharification process. Some glucoamylases have already been reported to be more active towards α-(1→6)-linkages than others (Fagerstrom & Kalkkinen, 1995). Also, properties like substrate binding or substrate specificity can be adapted by targeted mutations (Reilly, 1999). To screen the activity of various glucoamylases towards branched gluco-oligosaccharides, ideally, isolated branched gluco-oligosaccharides should be used (Jonathan et al., 2015). In this research, therefore, it was aimed to evaluate nine experimental glucoamylases for their mode-of-action towards a branched gluco-oligosaccharide with DP5, officially named as glc(α1-4)[glc(α1-4)glc(α1-6)]glc(α1-4)glc (McNaught, 1996). In addition, it was assessed whether commercially available substrates such as panose (glc(α1-6)glc(α1-4)glc) and pullulan could represent branched gluco-oligosaccharides in enzyme screening.

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2. Materials and methods

2.1. Materials

The reference enzyme (GA1) was purified from a commercial enzyme cocktail Distillase® SSF (DuPont Industrial Biosciences, Leiden, The Netherlands). The latter commercial cocktail was not used further in this research. GA1 was developed by introduction of 5 mutations on a previously characterised glucoamylase from *H. jecorina* (Bott et al., 2008). The evaluated glucoamylases were homologs of GA1 (GA2 and GA3) or experimental glucoamylases with 3–6 additional mutations on GA1 (GA4–GA10). All enzymes were purified and supplied by DuPont Industrial Biosciences. All other chemicals, when not mentioned specifically, were of analytical grade and obtained from Sigma–Aldrich (Steinheim, Germany).

2.2. Enzyme activity towards commercial substrates

The enzyme activity towards commercial substrates was assessed by monitoring the amount of β-glucose released from the substrates by the inverting glucoamylases using an adapted colorimetric assay. In principle, β-glucose was oxidised by glucose oxidase to gluconic acid. With horse radish peroxidase (HRP) as catalyst, the hydrogen peroxide that was released during the reaction oxidised ABTS, producing green colour (Okuda, Miwa, Maeda, & Tokui, 1977). The commercial substrates used were maltose, maltotriose, maltoheptaose, isomaltose, panose (glc(α1-6)glc(α1-4)glc); Sigma–Aldrich) and pullulan (Fluka, Zwijndrecht, The Netherlands). The purity of all commercial substrates used were >97%. The details of the adapted procedure are described below.

The enzyme mixture (Reagent A) contained 25 units of HRP type VI (Sigma–Aldrich), 62 units/mL glucose oxidase (OxyGo HPL5000; DuPont Industrial Biosciences), 50 mM sodium acetate buffer pH 4.3 and 0.005% (v/v) Tween 80. One unit of HRP type VI was defined as the amount that would produce 1 mg purpurogallin from pyrogallol in 20 s at pH 6.0 at 20 °C. One unit of OxyGo HPL5000 was defined as the amount that would oxidise 3 mg glucose to gluconic acid in one minute at pH 5.1 at 35 °C. The substrate mixture (Reagent B) contained 9.8 mM ABTS (Sigma–Aldrich), 100 mM commercial substrate, 50 mM sodium acetate buffer pH 4.3 and 0.005% (v/v) Tween 80. For pullulan, the substrate concentration in reagent B was 4%(w/v). An aliquot of 40 μL reagent A was mixed with 15 μL glucoamylase (30 μg/mL) and 50 μL reagent B in a microtitre plate. The absorbance at 405 nm (A_{405}), which represented the amount of β-glucose released by the glucoamylase, was monitored every 9 s for 3.5 min during incubation at 25 °C. The performance index of an experimental enzyme towards a substrate was calculated from the ratio between the increase rate of A_{405} for the experimental enzyme and that for GA1.

2.3. Enzyme activity towards branched gluco-oligosaccharide DP5 (bDP5)

The substrate used to assess glucoamylase activity towards bDP5 was a size exclusion chromatography fraction rich in branched gluco-oligosaccharide with a degree of polymerisation of 5 (bDP5; glc(α1-4)[glc(α1-4)glc(α1-6)]glc(α1-4)glc; purity ~84%(w/w)) that was produced, isolated and characterised as described elsewhere (Jonathan et al., 2015). The reaction mixture contained 500 μg/mL of bDP5-rich fraction and 5 μg/mL enzyme protein in 100 mM sodium acetate buffer pH 4.85. Aliquots of 50 μL were taken after 30 min, 1 h, 2 h, 4 h, 8 h and 24 h of incubation at 32 °C. Enzymes were inactivated by heating the samples in a boiling water bath for 10 min. Samples were diluted ten times for glucose, maltose and oligosaccharide profiling and quantification by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) following a procedure described elsewhere (Jonathan et al., 2015). Glucose, maltose, maltotriose, maltotetraose, maltopentaose, isomaltose and panose (all from Sigma–Aldrich) were used as standards. Calibration curves were prepared by analysing standard mixtures containing 5, 15, 25, 40 and 50 μg/mL of each of the compounds in the same run as the samples. The bDP5 and branched DP4 (bDP4; glc(α1-4)[glc(α1-6)]glc(α1-4)glc), which is an intermediate degradation product of bDP5, were quantified based on response factors obtained for maltopentaose and maltotetraose, based on the assumption that the dose-response relationship of bDP5 and bDP4 were similar to those of maltopentaose and maltotetraose, respectively. The performance index of an experimental enzyme towards bDP5 was calculated based on the ratio of the amount of glucose and maltose released after 2 h incubation by the experimental enzyme and the amount released by GA1.

3. Results and discussion

3.1. Enzyme activity towards commercial substrates

The activity of GA1 purified from Distillase® SSF was used as reference for the other experimental enzymes. The amount of β-glucose released per minute by GA1 from maltotriose and maltoheptaose was three times and four times higher, respectively, than that released from maltose. This result suggested that this enzyme had higher activity on larger oligosaccharides. Its activity towards α-(1→6) linkage in isomaltose was ten times lower than that towards α-(1→4) linkage in maltose. The activity towards panose (glc(α1-6)glc(α1-4)glc) was similar to that towards maltose and its activity on pullulan was half of the activity towards maltose.

The performance index of the experimental enzymes towards commercial substrates compared with that of GA1 is shown in Table 1. From this table, it was seen that the activity of the experimental glucoamylases towards α-(1→4)- and α-(1→6)-linkages varied. The activity of enzyme GA7 towards the tested commercial substrates was similar to that of GA1. Enzymes GA2 and GA3 had the highest activity on pullulan, with comparable activity to GA1 on the other substrates. The higher activity on pullulan indicated that these enzymes had higher activity on α-(1→6)-linkage. Nevertheless, the performance index on panose was not as high as that for pullulan, suggesting that compared with GA1, GA2 and GA3 had higher preference to larger molecules as their substrates. Compared to GA1, enzyme GA4 and GA5 seemed to be more active on α-(1→6)-linkage and have higher preference to shorter oligosaccharides. Enzyme GA6 had similar activity to GA1 towards α-(1→4)-linkages, but slightly higher activity for α-(1→6) linkages. Enzyme GA8 had higher activity than GA1 for maltoheptaose, but it had lower activity for the other substrates, indicating that it has higher preference for longer α-(1→4)-linked gluco-oligosaccharides. Enzyme GA9 and GA10 had the highest activity on isomaltose and panose, but this high activity seemed to be obtained at the expense of the activity towards the tested linear α-(1→4)-linked gluco-oligosaccharides. GA9 and GA10 were also less active than GA1 towards pullulan, probably because of the low activity towards α-(1→4)-linkage.

The results of Table 1 may reflect the relative activity of glucoamylases towards α-(1→4)- and α-(1→6)-linkages, but none of the commercial substrates tested had the branched structure [4)Glc(α1-4)[4)Glc(α1-6)]Glc(α1-] like the one present in branched gluco-oligosaccharides. Nevertheless, the branched gluco-oligosaccharides were also built of glucosyl residues with α-(1→4)- and α-(1→6)-linkages. Hence, it was hypothesised that glucoamylases with a high performance on panose or

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