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Structure of branching enzyme- and amylomaltase modified starch produced from well-defined amylose to amylopectin substrates

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ABSTRACT

Thermostable branching enzyme (BE, EC 2.4.1.18) from *Rhodothermus obamensis* in combination with amylomaltase (AM, EC 2.4.1.25) from *Thermus thermophilus* was used to modify starch structure exploring potentials to extensively increase the number of branch points in starch. Amylose is an important constituent in starch and the effect of amylose on enzyme catalysis was investigated using amylose-only barley starch (AO) and waxy maize starch (WX) in well-defined ratios. All products were analysed for amylopectin chain length distribution, α-1,6 glucosidic linkages content, molar mass distribution and digestibility by using rat intestinal α-glucosidases. For each enzyme treatment series, increased AO content resulted in a higher rate of α-1,6 glucosidic linkage formation but as an effect of the very low initial branching of the AO, the final content of α-1,6 glucosidic linkages was slightly lower as compared to the high amylopectin substrates. However, an increase specifically in short chains was produced at high AO levels. The molar mass distribution for the enzyme treated samples was lower as compared with substrate WX and AO, indicating the presence of hydrolytic activity as well as cyclisation of the substrate. For all samples, increased amylose substrate showed decreased α- and β-amylolysis. Surprisingly, hydrolysis with rat intestinal α-glucosidases was higher with increasing α-1,6 glucosidic linkage content and decreasing $\overline{M}_w$ indicating that steric hindrance towards the α-glucosidases was directed by the molar mass rather that the branching density of the glucan per se. Our data demonstrate that a higher amylose content in the substrate starch efficiently produces α-1,6 glucosidic linkages and that the present of amylose generates a higher $\overline{M}_w$ and more resistant product than amylopectin. The combination of BE → AM → BE provided somewhat more resistant α-glucan products as compared to BE alone.

1. Introduction

The biosynthesis of starch and glycogen are complex processes consisting of a multitude of enzyme catalyzed transfer and hydrolysis reactions. In general, starch consists of two main components: amylose and amylopectin. Amylose is composed of long and mainly linear backbone α-glucan chains linked together by α-1,4 glucosidic linkages while amylopectin is a branched component linked together by α-1,4 glucosidic backbone with α-1,6 glucosidic branch points (Pérez and Bertoft, 2010). For the industry, such branching pattern is important and starches are selected and further modified to enhance its versatility and satisfy consumer demand (Tharanathan, 2005). Typically, increased branch density can increases solubility of the starch and can suppress amylolytic hydrolysis providing health-associated functionality

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(Sorndech et al., 2015). However, such glycogen-like functionality is not available in pure bulk quantity from starch crops.

Enzymatic modification of starch is gaining an interest as a clean and safe alternative to chemical modification (Blennow et al., 2013). Such enzymatic starch modification may be carried out either by the use of recombinant enzymes acting on purified starches, or alternatively the enzymes may be expressed directly in the starch producing crops by GM technology (Hebelstrup, Sagnelli, & Blennow, 2015). However, the GM crop approach may sometimes modify the starch to an extend where its function as a storage of biomass and energy is jeopardized so that biological functions such as grain germination and seedling establishment are deficient (Shaik, Carciofi, Martens, Hebelstrup, & Blennow, 2014). Amylomaltase (AM, 4-α-D-α-glucanotransferase; E.C. 2.4.1.25. glucosyl hydrolase family 13 and 70, GH13 and GH70, www.CaZy.org) transfers α-1,4 glucosidic linkages mainly within amylopectin and amylose, amylopectin cluster, or from amylose to amylopectin (Hansen, Blennow, Pedersen, Nørgaard, & Engelsen, 2008). AM has been proven to add value to starch including its modification to impose gelatin-like functionality (Hansen et al., 2008) and increased degradative resistance (Jiang, Miao, Ye, Jiang, & Zhang, 2014). Branching enzyme (BE, 1,4-α-D-α-glucan:1,4-α-D-α-glucan 6-α-D-(1,4-α-D-α-glucano)-transferase, EC 2.4.1.18, glucosyl hydrolase family 13, GH13, www.CaZy.org) catalyses the formation of branch points in starch and glycogen biosynthesis by the cleavage of existing α-1,4 glucosidic linkages followed by transfer to 1,6 hydroxyl groups to form the tree-like amylopectin and glycogen structures (Roussel et al., 2013; Shinohara et al., 2001). In vitro, BE also catalyses a cyclisation reaction to form cyclo-amylose and cylo-amylopectin clusters (Kelly, Dijkhuizen, & Leemhuis, 2009; Takata, Takaha, Okada, Hizukuri et al., 1996). Starch modification with BE demonstrates an increase in solubility, reduced viscosity and for AM, increase degradative resistance of the product (Blennow et al., 2013). The thermostable BE from *R. obamensis* has drawn interest in starch modification to efficiently produce highly-branched α-glucans (Roussel et al., 2013; Shinohara et al., 2001). Both amylose and amylopectin have been tested as substrate to identify possible different mechanisms. Some evidence suggests that *R. obamensis* BE activity was higher towards amylose than amylopectin (Shinohara et al., 2001). However, the substrate used was amylose from starch fractionation (Lee et al., 2008) which had amylopectin contamination or was enzymatic synthesized glucans containing DP 2–60 (Roussel et al., 2013), none of which the chain length was long enough compared to natural amylose. Different types of natural starch differ in their amylose content but there is only limited information on the effect of amylose on BE catalyzed formation of α-1,6 glucosidic linkages and cyclo-glucans (Andersson, Rydberg, Larsson, Andersson, & Åman, 2002; Roussel et al., 2013; Takata et al., 1997; Takata, Takaha, Okada, Takagi, & Imanaka, 1996). In a previous study, we modified cassava starch by using combinations of AM and BE to increase the content of α-1,6 branch points. The results showed that the highest content of α-1,6 branching points was achieved when using specific serial combinations of AM and BE.

In the present work we investigate the effects of amylose ratios of the substrate starch on AM and BE catalysis. As model substrates, we use the transgenic barley amylose-only (AO) starch which consists of more than 99% amylose (Carciofi et al., 2012) and 100% amylopectin waxy (WX) maize starch in well-defined ratios. The efficiency of α-1,6 branch formation for BE only and sequential BE → AM → BE treatment was investigated and the molecular structures and amylolytic digestibility analysed in vitro using rat intestinal α-glucosidases. The study provides additional information to better understand how BE only and optimized combinations of BE and AM treatments affect the structure and digestibility of the enzyme-modified α-glucan.

2. Materials and methods

2.1. Materials

Waxy maize starch (WX) was obtained from Cerestar-AKV I/S (Vodskov, Denmark). Amylose-only (AO) barley starch was obtained from Aarhus University (Aarhus, Denmark). BE, AM and β-glucanase were kindly provided from Novozymes (Bagsvaerd, Denmark). For BE and AM, one U is defined as 1 μmole/min under standard conditions. Isoamylase (EC 3.2.1.68, specific activity 210 U mL^{-1}) and β-amylase (EC 3.2.1.2, specific activity 620 U mL^{-1}) was obtained from Megazyme (Wicklow, Ireland). Porcine pancreatic α-amylase (EC 3.2.1.1, specific activity 22 U mg^{-1}), and glucoamylase from *Aspergillus niger* (EC 3.2.1.3, specific activity 129 U mg^{-1}) were purchased from Sigma-Aldrich (Missouri, USA). Proteinase K, recombinant, PCR grade was purchased from Roche (Hvidovre, Denmark). Enzyme activity units of isoamylase, α-amylase and glucoamylase are given according to the supplier.

2.2. AO barley starch extraction

Amylose-only barley grains (Carciofi et al., 2012) were ground into fine powder and 200 g of flour were mixed in 2 L of 1 mM DTT (dithiotreitol), and 1% SDS (sodium dodecyl sulfate) for 30 min while stirring. The pellet was collected by sediment the starch granules on ice for 1–2 days. The washing procedure was repeated once. The pellet was resuspended in 2 L of deionized water and the slurry sieved through a 70 μm sieve. To remove trace of cell-wall and protein contaminants, the granular starch preparation was subjected to β-glucanase and proteinase K treatments and the sedimented starch was washed 3 times in distilled water, once in 96% ethanol and finally air dried at room temperature.

2.3. Non-granular AO starch preparation

Non-granular starch was prepared according to Kong, Bertoft, Bao, and Corke (2008). Granular AO starch (5 g) was dissolved in 100 mL of 90% DMSO (dimethyl sulfoxide) by heating the mixture in a boiling water bath with constant stirring for 3 h. The AO slurry was placed at room temperature and 200 mL of 95% ethanol was added with continuous stirring. A further 200 mL of 95% ethanol was added, the slurry was left at room temperature and then centrifuged at 2500*g* for 10 min. The precipitate was suspended with 25 mL of 95% ethanol and pelleted at 2500*g* for 10 min. The washing procedure was repeated once with 95% ethanol and finally with acetone. The final non-granular AO precipitate was freeze-dried (Kong et al., 2008).

2.4. Enzymatic modification

2.4.1. BE action on substrates with well-defined amylose:amylopectin ratios

BE-modified starch was produced mainly as described (van der Maarel et al., 2005) with slight modifications. The WX and AO mixtures (2% wv^{-1}) with non-granular AO content varied from 0, 20, 50, 80 and 100% (0%AO, 20%AO, 50%AO, 80%AO and 100%AO) were suspended in 50 mM phosphate buffer, pH 6.5. The suspension was heated to 120 °C in an oil bath for 3 h while stirring by magnetic stirrer then cooled to 80 °C for 2 min. BE (4000 U g $starch^{-1}$) was added to the gelatinised starch paste and incubated at 80 °C for 30 min, then 60 °C for 24 h. The reaction was terminated by heating in boiling water bath for 30 min. The denatured protein and trace insolubles were removed by centrifugation (1500*g* for 20 min). The supernatant containing the soluble α-glucan product was recov-