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Synergistic amylomaltase and branching enzyme catalysis to suppress cassava starch digestibility

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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.

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, Warren, Grassby, Patel, & Ellis, 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

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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 attachment of 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 amylose and amylopectin (Takata et al., 1996a). 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 et al., 1996a). Very recently, BE was demonstrated to possess a minor transfer 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 et al. (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 3 h or 24 h 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 (4000 U/g starch) was added and the reaction mixture incubated at 60 °C for 24 h. The reaction was terminated by heating in boiling water bath for 30 min, trace insolubles removed by centrifugation (1500 × *g* 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 (4000 U/g starch) was added and the mixture incubated at 60 °C for 24 h. 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 3 h or 24 h. 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 (4000 U/g starch) were added and the mixture incubated at 60 °C for 3 h or 24 h. 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 (4000 U/g starch) were added and the mixture incubated at 70 °C for 3 h or 24 h. The product was collected and dried as above.

2.3. Iodine complexation

Iodine colorimetric analysis was carried out mainly as described by Wickramasinghe, Blennow, & Noda, 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 (1200 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 I_2 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 et al., 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