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Investigation of debranching pattern of a thermostable isoamylase and its application for the production of resistant starch

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ABSTRACT

Debranching enzymes contribute to the enzymatic production of resistant starch (RS) by reducing substrate molecular weight and increasing amylose yield. In the present study, the action pattern of a thermostable isoamylase-type debranching enzyme on different types of starch was investigated. The molecular weight distribution, glycosidic bond composition and contents of oligosaccharides released were monitored by various liquid chromatography techniques and nuclear magnetic resonance spectroscopy (NMR). These analyses showed that the isoamylase could specifically and efficiently attack α-1,6-glucosidic linkages at branch points, leaving the amylose favored by other amylolytic enzymes. Its ability to attack side chains composed of 1–3 glucose residues differentiates it from other isoamylases, a property which is also ideal for the RS preparation process. The enzyme was used as an auxiliary enzyme in the hydrolytic stage. The highest RS yield (53.8%) was achieved under the optimized conditions of 70 °C and pH 5.0, using 7 U isoamylase per g starch and 2 NU amylase per g starch. These data also help us better understand the application of isoamylase for preparation of other products from highly branched starch materials.

1. Introduction

Starch is classified as rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) [1] according to the rate and extent of its digestion. Due to its unique characteristic of being "resistant" to hydrolysis by α-amylase, RS has the benefits of decreasing plasma glucose and insulin, and it leads to increased fecal bulk and more short-chain fatty acid production by fermentation in the large intestine, which improves the control of both obesity and diabetes and consequently reduces the risk of cardiovascular disease [2,3]. Moreover, as a functional food ingredient, RS has desirable physicochemical properties such as white color, bland flavor, gel formation, viscosity elevation, and water-binding capacity [4,5]. As a result of this, RS has attracted increasing attention from nutritionists in recent years.

RS can be divided into four fractions, according to their resistance to digestion. RS1 is physically inaccessible starch, RS2 is native granular starch, RS3 is retrograded starch, and RS4 is chemically modified starch [6,7]. Among industrially produced RS, RS3 produced by the retrogradation of amylose by cooling gelatinized starch makes up the largest part [8,9]. At present, RS is mainly manufactured from high-amylose starch by physical methods including the heating-cooling process and chemical modification, with an unsatisfactory yield of 20–30%. That process may also have food safety problems [10]. In contrast, the enzymatic method or a combined enzymatic and physical method can reduce the substrate requirement for RS production and help avoid the safety issues in food-grade RS production. Recent studies have indicated that the use of amylose with lower molecular mass can increase the yield of retrograded starch after gelatinization. Based on the above understanding two enzymes, α-amylase and pullulanase, are currently employed for molecular mass reducing and debranching purposes. Pullulanase has been approved as an effective auxiliary hydrolytic enzyme for processing different materials for RS enzymatic production by Liu and co-workers [11]. Haripriya compared the amylose and RS content of retrograded starch before and after treatment with pullulanase (40 U per g starch) using 10% (w/w) red kidney bean starch as substrate. The results showed that the

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enzyme could improve RS yield by 34% (from 31.47% to 42.34%), which is nearly proportional to the increase in amylose content (39%) [12]. Jin treated 20% (w/w) maize starch with thermostable α-amylase before pullulanase debranching and obtained an even higher RS yield, 58.87%, after repeated enzyme hydrolysis and retrogradation [13]. As the commercial pullulanase was not stable above 50 °C, a cooling step was necessary after α-amylase treatment.

Like pullulanase, isoamylase (EC 3.2.1.68, glycogen-6-glucanohydrolase) could hydrolyze α-1,6-glucosidic linkages in amylopectin to yield amylose and oligosaccharides [14,15]. The two debranching enzymes can be distinguished by different substrate specificities, while their mechanisms of action are not thoroughly understood. One of the differences between the two debranching enzymes, suggested by Liu, is that pullulanase can cleave smaller side chains (maltose or maltotriose) [11], which is an advantage over isoamylase for the application of RS production. On the other hand, isoamylase is more tolerant of high temperatures. The molecular mass reduction and debranching processes can be performed simultaneously at higher temperatures to increase the reaction rate, which in turn decreases the amount of enzyme needed. The use of higher temperatures also inhibits microbial contamination. Therefore, exploring the application of thermostable isoamylase in the production of RS is of great value.

In previous studies, an isoamylase from a thermophilic bacillus strain was purified and characterized, showing excellent thermostability at 70 °C and neutral pH [16]. There was a regrettable lack of information about the manner in which this thermostable enzyme carried out the hydrolysis of starch. To explore the possibility of applying this enzyme to RS production, the present study investigated the debranching properties of the thermostable isoamylase. Based on the characteristics revealed, the hydrolyzing conditions for RS production using isoamylase and thermostable α-amylase were also optimized.

2. Material and methods

2.1. Materials

Amylopectin and amylose were obtained from Fluka (Buchs, Switzerland). Soluble starch and chemical reagents were obtained from Sinopharm Chemical Reagent (Shanghai, China). Maize starch, potato starch and waxy rice starch were bought from local markets. α-Amylase (BAN 480 L, 480 KNU g^{-1}), amyloglucosidase (AMG 300 L 300 AGU g^{-1}) and protease (Neutrase 0.8 L, 0.8 AU g^{-1}) were obtained from Novozymes (Bagsvaerd, Denmark). Recombinant isoamylase was produced by *E. coli* and purified as in previous studies; the encoding gene originated from a thermophilic bacterium, *Bacillus lentus*. The purified monomeric protein with a molecular mass of 108 kDa displayed an optimal temperature and pH of 70 °C and 6.0, respectively, which made it ideal for starch processing. The purified enzyme had a specific activity of 6535 U mg^{-1} (using 2% glycogen as substrate, enzyme assay conditions were 50 °C, pH 6.0, reaction time was 30 min. 1 U = the amount of enzyme capable of producing an increase in absorbance of 0.01 at 600 nm in 1 h at 50 °C) [16].

2.2. High-performance size-exclusion chromatography (HPSEC)

HPSEC was used to monitor the changes in the molecular weight of isoamylase digested with maize amylopectin and amylose, which were treated under the following conditions. Samples (2 mg mL^{-1}) were first dissolved in 0.2 M acetate buffer (pH 6.0) and boiled for 10 min. Once the solution cooled to 60 °C, 2 U purified isoamylase was added and the 50-mL reaction mixture was incubated at 60 °C for 2 h. The HPSEC equipment consisted of an integrated autosampler and pump module (Waters 600, Waters, Milford, MA, USA) and a refractive index (RI) detector (Waters 2410), which were controlled by Waters Empower™ 2 Chromatography Software. An Ultrahydrogel™ Linear column (Waters, 300 mm × 7.8 mm) at a temperature of 45 °C was used for the water-based eluent. The flow rate was 0.9 mL min^{-1} and the injection volume was 20 μL. The HPSEC data were also processed with the Empower™ 2 software. Dextran standards with different molecular weights (T-2000, T-70, T-40, T-20, and T-10; Sigma, St. Louis, MO, USA) were to calibrate the column and establish a standard curve.

2.3. High-performance anion-exchange chromatography with pulsed amperometric detector (HPAEC-PAD)

Maize amylopectin was treated in the manner described above. HPAEC was then used to detect the branch-chain length distribution. The analysis was carried out on an Ion Chromatography System 5000 (Dionex Canada Ltd., Oakville, ON, Canada) consisting of an autosampler, a GP 40 gradient pump and an ED 40 electrochemical detector. Isoamylase digested samples were eluted according to the method described by Kool [17], using a CarboPac™ PA-1 (4 × 250 mm, Dionex, Idstein, Germany) anion-exchange column pre-fitted with a guard column.

2.4. Proton nuclear magnetic resonance spectroscopy (^1H NMR)

Dissolved maize starch (1 g) was exhaustively treated with 200 U of isoamylase at 60 °C to examine the specificity of the enzyme for α-1,4- and α-1,6 bonds. The chemical bond contents were analyzed using ^1H NMR. That analysis was performed using an AVANCE III 400 MHz Digital NMR Spectrometer (Bruker, Switzerland) according to the method of Lopez [18]. Samples were first dissolved in 1 mL of deuterium oxide (D_2O), incubated for 2 h at room temperature, and then lyophilized. The D_2O-treated samples were redissolved in 0.6 mL of D_2O, and ^1H NMR spectra were obtained at 65 °C. The ratio of α-1,4- to α-1,6 glucosidic linkages was determined by integration of the anomeric resonance at 5.4 and 5.0 ppm, respectively.

2.5. Oligosaccharide quantitative analysis

Five substrates (amylopectin, soluble starch, maize starch, potato starch, and waxy rice starch) were treated in the manner described in HPSEC experiment. In order to prove whether the enzyme possesses the ability to cleave α-1,6 bonds on terminal glucose residues, two substrates, Panose (Promega) and mixed linkage amylosaccharide standards (prepared in this study, Fig. S1) were treated with the purified isoamylase (20 U per g starch at 70 °C and pH 6.0 for 4 h). The concentrations of glucose, maltose and other oligosaccharides were determined by an Agilent 1200 HPLC instrument (Santa Clara, CA, USA) under the following conditions: column, Waters XBridge Amide 5 μm 4.6 × 250 mm column (Millford, MA, USA); solvent, acetonitrile: water (65:35); flow rate, 1 mL min^{-1}; column temperature, 40 °C. The reaction mixture (0.5 mL) was added to an equal volume of acetonitrile, centrifuged at 10,000 *g* for 10 min at room temperature, filtered through a 0.45-μm filter and injected with a 20-μL loop.

2.6. Preparation of RS

RS was prepared in two steps, an enzyme hydrolysis and an autoclave cooling, according to the methods described by Kapelko [19] with some modifications. In the first step, 1 L maize starch slurry (15% w/v) was heated to 70 °C with stirring for 30 min. Next,