



Preparation of linear maltodextrins using a hyperthermophilic amylopullulanase with cyclodextrin- and starch-hydrolysing activities



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ABSTRACT

A novel method for the preparation of linear maltodextrins from cyclodextrins and starch was proposed. To accomplish this process, an amylopullulanase from hyperthermophilic archaeon *Caldivirga maquilgensis* (CMApu) was characterized and used. CMApu with an estimated molecular mass of 62.7 kDa by SDS-PAGE had a maximal pullulan-hydrolysing activity at 100 °C and pH 5.0. It could also hydrolyse amylopectin (AP), starch, β -CD and amylose (AM), in a decreasing order of relative activities from 88.96% to 57.17%. TLC and HPAEC analysis revealed that CMApu catalyzed the debranching and degrading reactions to produce linear malto-oligosaccharides (\leq G8–G1) from G8- β -CD and/or normal CDs, amyloextrins (DP6–96) from AM, and amyloextrins (DP1–76) from AP and potato starch. Our results showed that CMApu had a great potential for the industrial preparation of linear maltodextrins from normal starch instead of waxy starch, malto-oligosaccharides or sucrose. And the high optimal temperature of CMApu facilitated the simultaneous gelatinization and hydrolysis of cereal starch.

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1. Introduction

Maltodextrins with the properties of moderate sweet, rapidly absorption and hygroscopicity are widely used in numerous foods (Chronakis, 1998; Fisher-Wellman & Bloomer, 2010; Kendig, Lin, Beilharz, Rooney, & Boakes, 2014). Linear maltodextrins, composed of a mixture of polymers of D-glucose units with α -1,4-glucosidic linkages, were classified by degree of polymerization (DP) into linear malto-oligosaccharides ($DP \leq 8$) and amyloextrins ($1 \leq DP < 17$ or more long). Compared with branched malto-oligosaccharides with α -1,6-glucosidic linkages besides α -1,4-glucosidic linkages, linear malto-oligosaccharides are more easily hydrolysed by human salivary or pancreatic α -amylase and mucosal α -glucosidase resulting in higher glycaemic index for nutrition and energy. Amyloextrins could reduce the amylose recrystallization of cereal foods (Xu et al., 2012) and improve the

stability of $\omega 3/\omega 6$ polyunsaturated fatty acids (Xu et al., 2013). They were non-swelling, insoluble, and usually included in the ingredient of tablets for a controllable release rate of drug (Steendam, Eissens, Frijlink, & Lerk, 2000; TeWierik, Eissens, Besemer, & Lerk, 1993).

Linear maltodextrins were often prepared by enzymatic hydrolysis of amylose, amylopectin, malto-oligosaccharides or transglycosylation of sucrose. These amylolytic enzymes, with different activities and action patterns, were classified into different families of glycoside hydrolases (GH) (Janeček, Svensson, & MacGregor, 2014). Human saliva, porcine pancreas, or *Bacillus subtilis* α -amylase of GH family 13 could attack the α -1,4-glucosidic linkages of amylose at 37–60 °C to produce amyloextrins directly (Jane & Robyt, 1984). The desired DP could be obtained by adding different amount of α -amylase in the reactions (Xu et al., 2012). *Bacillus acidopullulyticus* type I pullulanase of GH family 13 could specifically attack the α -1,6-glucosidic linkages of amylopectin. When it was incubated with waxy maize starch at 55 °C, the amyloextrins with a DP of 35 were formed by the debranching reaction of amylopectin (TeWierik, Eissens, Besemer, & Lerk, 1993). *Escherichia coli* amyloamylase (Pajatsch, Böck, & Boos, 1998) and *Saccharophagus degradans* 4- α -glucanotransferase (Hwang, Choi, Kim, & Cha, 2013) of GH family 77 at 35 °C could catalyse the disproportion reaction between maltose, maltotriose or maltodextrins

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to synthesize the longer linear maltodextrins. *Neisseria polysaccharaea* amylase of GH family 13 at 35 °C could catalyse the formation of linear maltodextrins with a DP of 35–48 by successive transfers of the glucosyl moiety of sucrose onto the released glucose in the presence of sucrose alone on the initial concentration of 600–100 mM (Potocki-Veronese et al., 2005). More recently, rabbit muscle phosphorylase- α was used at 37 °C to prepare linear maltodextrins with a DP of 6–89 by successive transfers of the glucosyl moiety of α -D-glucose 1-phosphate onto maltopentaose, maltohexaose, or maltoheptaose (Kazłowski & Ko, 2014). However, it is not suitable for these amylolytic enzymes to produce linear maltodextrins from normal cereal starch.

Amylopullulanase, a type II pullulanase, with the cleavage activity of both α -1,4- and α -1,6-glucosidic linkages in starch and other related oligosaccharides, is attractive to prepare the linear maltodextrins using the cheap starch as the only substrate by breaking the chains of amylose and debranching the side chains of amylopectin. Thermostable amylopullulanases are especially favorable in the hydrolysis of starch, as high temperatures often facilitate the simultaneous gelatinization of starch, accelerate the reaction rate, and decrease the possibility of microbial contamination.

Hyperthermophilic archaea with optimal growth temperatures between 80 and 110 °C have been considered as a source of genes encoding thermostable enzymes (Egorova & Antranikian, 2005; Vieille & Zeikus, 2001). The archaeal amylopullulanases of GH family 57 from *Thermococcus hydrothermalis* (Erra-Pujada, Debeire, Duchiron, & O'Donohue, 1999; Gantelet & Duchiron, 1998), *T. litoralis* (Imamura et al., 2004), *T. siculi* (Jiao et al., 2011), *Pyrococcus furiosus* (Dong, Vieille, & Zeikus, 1997; Rüdiger, Jorgensen, & Antranikian, 1995) and *Staphylothermus marinus* (Li, Li, & Park, 2013) have been cloned and expressed in mesophilic *E. coli* successfully. All these recombinant enzymes have the maximal activities at their optimal temperatures between 85 and 105 °C, indicating the extremely thermostable properties.

Caldivirga maquilensis is an anaerobic, sulfur-dependent, rod-shaped, and hyperthermoacidophilic Crenarchaeota isolated from an acidic hot spring in the Philippines (Itoh, Suzuki, Sanchez, & Nakase, 1999). This microorganism has a high amount of tetraether core lipids and trace amounts of diether core lipids to help it thrive within its very hot (60–92 °C) and very acidic (pH 2.3–6.4) environment. Genomic sequencing of *C. maquilensis* has revealed that its some genes might be related to the hydrolysis of starch and transportation (Copeland et al., 2007).

In this study, a gene encoding a protein of GH family 57 from *C. maquilensis* was cloned and expressed in *E. coli*. Characterization of the biochemical properties of the purified recombinant protein revealed that it was an extremely thermostable amylopullulanase with CD/AM-degrading and long branched cyclodextrin (LBCD)/AP-debranching activities. The results provided a novel method for the production of linear maltodextrins from LBCD or normal cereal starch.

2. Materials and methods

2.1. Chemicals

Pullulan, potato starch (normal starch, amylose, and amylopectin), malto-oligosaccharides (from maltose to maltoheptaose; i.e. G2–G7), cyclodextrins (α -CD, β -CD, and γ -CD), and short branched cyclodextrins (6-O-glucosyl- β -CD and 6-O-maltosyl- β -CD; i.e. G1- β -CD and G2- β -CD) were purchased from Sigma (St. Louis, MO, USA). Long branched cyclodextrin (6-O-maltooctosyl- β -CD; i.e., G8- β -CD) was kindly provided by Professor Kwan-Hwa

Park (Department of Foodservice Management and Nutrition, Sangmyung University, Republic of Korea). All restriction enzymes, calf intestinal alkaline phosphatase, T4 DNA ligase, and PrimeSTAR[®] HS DNA Polymerase were supplied by Takara Biotechnology (Dalian, Liaoning, China). All other chemicals used were of reagent grade and were purchased from Sinopharm Chemical (Shanghai, China). Double distilled water was prepared with a distilled water system (BSZ-2; Botonyc, Shanghai, China). Deionized water was prepared with an ultra-pure water system (Molelement 1820a; Molecular, Shanghai, China).

2.2. Cloning and expression of the gene encoding CMApu

The gene encoding CMApu was amplified from *C. maquilensis* IC-167 genomic DNA (kindly provided by Professor Christopher Howard House, Department of Geosciences, Pennsylvania State University, USA) by PCR using PrimeSTAR[®] HS DNA Polymerase at an annealing temperature of 55 °C (G-Storm Thermal Cycler; GRI, Braintree, Essex, UK). CMApu gene-specific primers flanking the 5' and 3' ends of Cmaq.1371 were designed based on the complete genome sequence of *C. maquilensis* IC-167 (Copeland et al., 2007). The forward (Cmaq.1371-NdeI, 5'-CATTCATATGGTCTACGTGAGGGCTTACTTGATG-3') and reverse (Cmaq.1371-XhoI, 5'-AGCCTCGAGCGCTGAATCCTCATTAGGT-3') primers (Takara Biotechnology) contained NdeI and XhoI restriction sites (underlined), respectively. The amplified fragment (1.82 kb) was digested with NdeI and XhoI, and then ligated into the expression vector pTKNd6xH (kindly provided by Professor Kwan-Hwa Park) with a BLMA promoter (Kim et al., 2003). The recombinant plasmid was designated pCMApu6xH. The sequence of inserted Cmaq.1371 was confirmed using dideoxy chain termination sequencing with an Applied Biosystems[®] 3730xl DNA Analyzer (Life Technologies, Foster City, CA, USA).

2.3. Production and purification of recombinant CMApu

E. coli MC1061 cells transformed with pCMApu6xH were cultured for 20 h at 250 rpm 37 °C in 6 flasks containing 250 mL of Luria-Bertani broth [1% (w/v) tryptone (Oxoid, Basingstoke, Hampshire, UK), 0.5% (w/v) yeast extract (Oxoid), and 0.5% (w/v) NaCl] supplemented with kanamycin (80 μ g/mL). The cells were then collected by centrifugation at 9000 \times g and 4 °C for 20 min (Avanti[®] J-26XP Centrifuge; Beckman Coulter, Brea, CA, USA), resuspended in 150 mL of binding buffer (20 mM sodium phosphate, pH 7.4, 500 mM NaCl, and 5 mM imidazole), and sonicated 5 times for 3 min each for 50 mL each of resuspended *E. coli* in an ice bath using an ultrasonic processor with a 1/2" (13 mm) probe with replaceable tip at net power output 750 W, frequency 20 kHz, amplitude 35%, pulse on 2 s and pulse off 1 s (VCX 750; Sonics & Materials, Newtown, CT, USA). The cell-free supernatant (163 mL) was then collected by centrifugation at 12,000 \times g and 4 °C for 20 min, and then heated at 70 °C for 15 min to remove all thermolabile proteins. The crude enzyme from heat treatment (154 mL) was further purified by an ÄKTApurifier UPC 10 system with a Ni Sepharose[™] 6 Fast Flow column (2.6 cm \times 10 cm) (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The active fractions in elution buffer (20 mM sodium phosphate, pH 7.4, 500 mM NaCl, and 500 mM imidazole) were concentrated and dialyzed by ultrafiltration (Amicon stirred cell model 8400; Merck Millipore, Billerica, MA, USA) against 20 mM sodium phosphate buffer (pH 7.4). The protein concentration was determined according to the Bradford method, using bovine serum albumin as a standard (Bradford, 1976). The purity and molecular weight of the protein were analysed by discontinuous SDS-PAGE (Laemmli, 1970).

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