High-yield cycloamylose production from sweet potato starch using *Pseudomonas* isoamylase and *Thermus aquaticus* 4- α -glucanotransferase

Sun Chu, Jung Sun Hong, Shin-Joung Rho, Jiyoung Park¹, Sang-Ik Han², Young-Wan Kim³, and Yong-Ro Kim*

Center for Food and Bioconvergence, Department of Biosystems and Biomaterials Science and Engineering, Seoul National University, Seoul 08826, Korea

¹Department of Central Area Crop Science, NICS, Suwon, Gyeonggi 16613, Korea

²Department of functional Crop, Functional Crop Resource Development Division, NICS, RDA, Miryang, Gyeongnam 50424, Korea

³Department of Food and Biotechnology, Korea University, Sejong 30019, Korea

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*Corresponding Author
Tel: +82-2-880-4607
Fax: +82-2-873-2049
E-mail: yongro@snu.ac.kr

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Abstract An optimal reaction condition for producing cycloamylose (CA) from sweet potato starch was investigated using a combination of isoamylase (from *Pseudomonas* sp.) and 4- α -glucanotransferase (from *Thermus aquaticus*, TA α GT). Starch was debranched by isoamylase for 8 h and subsequently reacted with TA α GT for 12 h. The yield and purity of CA products were determined using HPSEC and MALDI-TOFMS, respectively. Consequently, the maximum yield was 48.56%, exhibiting the highest CA production efficiency ever reported from starch. The CA products showed a wide range of the degree of polymerization (DP) with the minimum DP of 5. CA was also produced by simultaneous treatment of isoamylase and TA α GT. The yield was 3.31%, and the final products were contaminated by multiple branched and linear molecules. This result suggests that a former reaction condition (the sequential addition of isoamylase and TA α GT) is preferable for producing CA from sweet potato starch.

Keywords: starch modification, enzymatic conversion, transglycosylation, debranching enzyme, normal starch

Introduction

Sweet potato (*Ipomea batatas*) is one of the most important economic crops that is widely produced in the Asian and African regions. It significantly contributes as a food supplement in developing countries as well as a source of nutrition for good health, such as dietary fiber, minerals, vitamins, and antioxidants in food industries (1). In addition, sweet potato starch has gained increasing attention in industrial utilization as value-added food and bio-based products, including biodegradable plastics and bioethanol (2).

Cycloamyloses (CAs) are cyclic compounds that comprise α -1,4-linked D-glucoses, which are similar to cyclodextrins (CDs). CAs are more soluble and stable in cold water than CDs. Also, CAs can form inclusion complex with various types of guest molecules and impact on the properties of guest molecules through complex formation. CA has been used as an efficient artificial chaperone for protein refolding (3) and as a polysaccharide-based carrier in a gene delivery system (4). In addition, it was noted that CA improved the solubility and bioavailability of drug compounds (5,6). Despite the great

potential of CA as value-added biomaterials, the industrial applications of CA are still limited due to its high production cost.

CAs are traditionally produced via intramolecular transglycosylation catalyzed by 4- α -glucanotransferase (4 α GTase) using either amylose or intact starch polymers. Depending on the origin of enzymes and the type of substrates, the minimum degree of polymerization (DP) of CA varies from 5 to 22 (7–11). The synthetic potato amylose, essentially the α -1,4-linked linear glucans, has been the most prevailing substrate for CA production (7). However, utilizing an isolated amylose is highly expensive and time-consuming, which limits the mass production and industrial application of CA. Furthermore, using intact starch polymer that contains both amylose and highly branched amylopectin as a substrate, leads to low CA production yields. This is ascribed to the lack of 4 α GTase activity on α -1,6-glycosidic linkages (branching points).

A recent study has shown that the CA production yield was improved by a combined treatment of debranching enzyme (i.e., isoamylase) and 4 α GTase (11). First, starch was enzymatically debranched to liberate α -1,4-linked linear glucan chains from the

branched polymers. Then, 4 α GTase used these linear chains as intermediate substrates for cyclization reaction. Using this approach, Xu *et al.* (11) observed that the yield of CA produced from high amylose starch increased from 24.55 to 45.58% and from 27.40 to 47.25% in the water and in dimethylsulfoxide (DMSO) reaction systems, respectively. However, high-amylose starches generally require a higher gelatinization temperature (>130°C) than normal amylose starches (12). Moreover, gelatinized high-amylose starches tend to form strong gels and have relatively high viscosities compared to normal amylose starches (13), which are the unfavorable physical properties for starch processing (13).

Therefore, the aim of this research was to develop an optimal enzymatic reaction condition for a high yield of CA production using sweet potato starch (SPS). In the present study, the enzymatic process was primarily designed with the sequential addition of isoamylase (from *Pseudomonas* sp.) and 4 α GTase (from *Thermus aquaticus*, TA α GT). Later, the effect of the simultaneous addition of those enzymes was compared. The quantity of CA products was analyzed by high-pressure size-exclusion chromatography (HPSEC), while the quality of the CA products were assessed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOFMS).

Materials and Methods

Materials and chemicals Sweet potatoes were provided by National Institute of Crop Science, Rural Development Administration (Jeonju, Korea). SPS was isolated from a Korean native sweet potato (*Yulmi*, Korea) by an alkaline method (14) with slight modifications. Cycloamylose (Avg. M.W.=7,500 Da) as a CA standard was purchased from Ezaki Glico Co., Ltd. (Osaka, Japan). Isoamylase (*Pseudomonas* sp., EC 3.2.1.68, 280 U/mg) and β -amylase (*Bacillus cereus*, 10,000 U/ml) were obtained from Megazyme International Ireland Ltd. (Wicklow, Ireland). Pullulan Shodex standard P-82 (P5, P10, P20, P50, P100, P200, P400, and P800) was purchased from Showa Denko (Tokyo, Japan). Other chemicals and reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and Duksan Pure Chemical Co., Ltd. (Ansan, Korea). All chemicals and reagents were at minimum of analytical grade unless indicated otherwise.

Preparation of TA α GT and enzyme activity assay The recombinant TA α GT (p6 $\times$ HisTA α GT-DE) was transformed into chemically competent *E. coli* MC 1061 for expression, as described by Park *et al.* (15), with slight modification. The transformed colonies were inoculated into the Luria-Bertani (LB) medium containing ampicillin (50 μ g/mL) and incubated in an orbital shaker (250 rpm) for 12 h at 37°C. The cells were collected by centrifugation (3,700 $\times g$ for 20 min at 4°C) and suspended in a 300-mL lysis buffer [50 mM Tris-HCl (pH 7.5), 300 mM NaCl, and 10 mM imidazole]. The cell extract was disrupted by sonication (VCX 750; Sonics & Materials Inc., Newtown, CT, USA) with

70% of the maximum amplitude and on/off pulse for 1 s each, while it was incubated for 20 min at 65°C. The supernatant was purified using a nickel-nitrilotriacetic acid (Ni-NTA) affinity column (1.6 cm $\times$ 2.5 cm; GE Healthcare, Uppsala, Sweden). The molecular mass of the TA α GT enzyme was estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (16). The protein concentration was measured according to the Bradford method (17). The enzyme activity was determined according to the method described by Liebl *et al.* (18). This assay is based on a change in iodine-staining property as amylose was converted by the enzyme in the presence of maltose. One unit of the enzyme was defined as the amount of enzyme which degrades 0.5 mg/mL of amylose per min.

Determination of enzymatic reaction conditions To determine an initial concentration of starch substrate and an optimal reaction time for isoamylase treatment (5 U/g, starch weight basis), the content of reducing sugar (19), the amount of linear α -1,4-glucans (%) (20), and molecular weight distribution (high-performance size-exclusion chromatography, *infra*) were determined as a function of the reaction time (0–12 h). An optimal reaction time for TA α GT (100 U/g, starch weight basis) was likewise determined by the time course observation of molecular weight distribution (0–12 h). Based on the results from these analyses, CA was produced in the optimal reaction conditions as described below.

Production of CA by sequential treatment of isoamylase and TA α GT SPS (8%, w/v) dispersed in 50 mM sodium acetate buffer (pH 4.5) was heated in a 95°C water bath for 30 min, followed by autoclaving at 121°C for 15 min. The gelatinized starch was cooled down and incubated with isoamylase (5 U/g, starch weight basis) for 8 h at 40°C via stirring. At the end of the reaction, the samples were boiled for 10 min to inactivate the enzyme activity. Subsequently, tenfold volume of 95% (v/v) ethanol was added to precipitate the debranched starches. The precipitates were collected by centrifugation (14,800 $\times g$, 10 min).

For subsequent treatment with TA α GT, the debranched starch (4%, w/v) was dissolved in 90% (v/v) DMSO by heating at 75°C for 30 min. Tris-HCl buffer (50 mM, pH 7.5) was added to obtain 1% (w/v) debranched starch solution. Subsequently, TA α GT (100 U/g, starch weight basis) was added. The starch solution was incubated at 75°C for 12 h via stirring. The samples were boiled for 10 min to inactivate the enzyme activity and centrifuged (14,800 $\times g$, 10 min) to remove the denatured enzyme proteins. The supernatant of starch solution was incubated with β -amylase (40 U/g) at 40°C for 12 h to degrade linear molecules into maltose. After boiling the sample for inactivating β -amylase, glucans resistant to β -amylase were precipitated by adding tenfold volume of 95% (v/v) ethanol and centrifugation (14,800 $\times g$, 10 min). The precipitate was washed twice with fivefold volume of 85 and 80% (v/v) ethanol, respectively. The final products were centrifuged (14,800 $\times g$, 10 min) and lyophilized.

Production of CA by simultaneous treatment of isoamylase and TA α GT

SPS (8%, w/v) was suspended in 50 mM sodium acetate buffer (pH 5) and gelatinized by stirring in a 95°C water bath for 30 min, followed by autoclaving at 121°C for 15 min. Debranching and cyclization reactions were initiated by adding both isoamylase (5 U/g) and TA α GT (100 U/g) at the same time. Subsequently, the starch-enzyme solution was incubated at 50°C for 8 h. The enzymes were inactivated by boiling for 10 min. The denatured enzyme proteins were removed by centrifugation (14,800 $\times$ *g*, 10 min). Then, the β -amylase treatment was conducted and the final products were collected in the same manner as described above.

High-performance size-exclusion chromatography (HPSEC)

The molecular weight distributions of starch branch chains and CA product were analyzed by HPSEC equipped with a pump (Prostar 210; Varian Inc., Palo Alto, CA, USA), injector (Rheodyne 7725i; Sigma-Aldrich), and refractive index detection system (Prostar 355 RI detector; Varian Inc.). The debranched starch was fractionated by passing through two SEC columns (Shodex OHpak SB-806 HQ and OHpak SB-804 HQ; 8.0 mm ID $\times$ 300 mm each; Showa Denko) at a flow rate of 0.4 mL/min. The CA product was separated by passing through two SEC columns (Shodex OHpak SB-804 HQ and OHpak SB-802.5 HQ columns; 8.0 mm ID $\times$ 300 mm each) at a flow rate of 0.8 mL/min. The commercial CA was dissolved in distilled water at various concentrations (0.1–1%, w/v) and injected onto HPSEC. A CA standard curve was obtained on the basis of the peak areas of different CA concentrations. The yield of CA product was determined using the CA standard curve. The molecular weight of CA product was assessed using the series of Pullulan Shodex standard.

Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOFMS)

The molecular masses of the CA products were determined using a MALDI-TOFMS spectrometer (Voyager^{TM}-DE; Perceptive Biosystem, Framingham, MA, USA) in reflection mode. The CA sample (1 μ g/mL) was mixed with a matrix (2,5-dihydroxybenzoic acid, 0.1–5 μ mol/ μ L) in equal volumes. The mixture (1 μ L) was added to the MALDI-TOFMS probe tip and air dried until homogeneous crystals formed. The dried sample was desorbed/ionized from the probe tip and analyzed under the following condition: accelerating voltage (20 kV), grid voltage (87%), grid wire voltage (0.3%), delayed extraction (300 ns), and nitrogen laser intensity (2000 shots).

Results and Discussion**Degree of debranching and the amount of linear α -1,4-glucans for debranched sweet potato starch**

The degree of debranching was represented as the content of reducing sugar, as the hydrolysis of α -1,6 glycosidic bond (branching point) produces reducing groups that bind with 3,5-dinitrosalicylic acid. As SPS was debranched, the

Table 1. The amount of linear α -1,4-glucans (%) of sweet potato starch (SPS) debranched by isoamylase depending on different reaction time

Debranching time (h)	Linear α -1,4-glucans (%) ¹⁾
0	21.07 $\pm$ 0.79
2	43.42 $\pm$ 1.01
4	51.22 $\pm$ 2.67
6	67.50 $\pm$ 5.48
8	63.93 $\pm$ 1.01
10	63.57 $\pm$ 3.82
12	65.00 $\pm$ 2.81

¹⁾The amount of linear α -1,4-glucans was obtained from calibration equation that was derived from the mixture of amylose and amylopectin from potato: $Y=0.0098X-0.0015$, where Y was assigned for absorbance at 620 nm and X was assigned for the amount of linear α -1,4-glucans.

degree of debranching rapidly increased during the initial 4 h and then slowly reached a plateau (data not shown). A complete debranching was achieved within 8 h. For 8 h of isoamylase reaction, the degree of debranching was proportional to the initial concentration of starch substrate up to 8% (w/v) of SPS. The amount of linear α -1,4-glucans liberated from SPS at various lengths of debranching time are depicted in Table 1. The initial amount of linear α -1,4-glucans for SPS was 21.07%, which is within a range of amylose contents (13.33–26.83%) reported for other SPS varieties (21). As the debranching time increased, this value gradually increased, indicating an increase in the linear α -1,4-glucan population capable of forming complex with iodine. In the initial 4 h of reaction, the hydrolysis appeared to proceed at a rapid rate, resulting in a sharp increase of linear α -1,4-glucans up to 67.50% of the total reaction; however, there was little change in the content thereafter. This result was consistent with the increasing trend in the amount of reducing sugars during the debranching period.

Molecular weight distribution of SPS treated with isoamylase and TA α GT

The molecular weight distribution profiles of either isoamylase- (Fig. 1) or TA α GT-treated SPS (Fig. 2) assessed by HPSEC were presented depending on the reaction time (0–12 h). In Fig. 1, the peak for amylopectin was eluted in a narrow range of retention time (R_T) 20–30 min, while that for amylose appeared as a broad peak across R_T 30–50 min. As SPS was debranched, the amylopectin peak gradually disappeared, while the third peak was gradually developed at R_T 40–50 min. The molecular weight of the formed glucans ranged from approximately 1.14×10^3 to 5.90×10^3 g/mol. After 8 h of the reaction, the third peak began to decline. It might be due to aggregation and precipitation of short linear chains released during the extended period of isoamylase debranching. It was reported that short linear chains (DP 6–80) tended to crystallize into A-type X-ray diffraction pattern during a late stage of debranching (4–24 h) (22,23), which might impede the cyclization reaction of 4 α GTase (24).

The effect of time for the TA α GT treatment on the CA production was investigated to optimize the reaction conditions. TA α GT is known to catalyze starch by transferring one α -1,4-glucan to another α -1,4-

Fig. 1. The molecular weight distribution of sweet potato starch (SPS) debranched by isoamylase depending on debranching time (0, 2, 4, 6, 8, 10, and 12 h).

Fig. 2. The molecular weight distribution of SPS degraded by TAαGT depending on reaction time (0, 2, 6, 8, and 12 h).

Fig. 3. Molecular weight distributions of cycloamylose (CA) standard. (A) CA products formed by sequential treatment of isoamylase and TA α GT, (B) reaction products formed by the simultaneous treatment of isoamylase and TA α GT, and (C) the additional isoamylase treatment of reaction products formed by the simultaneous treatment of isoamylase and TA α GT.

glucan via disproportionation (15). While the SPS was reacted by TA α GT, the change in the molecular weight distribution was monitored via HPSEC over the course of reaction period (0–12 h) (Fig. 2). As the reaction proceeded, the peak for amylopectin at R_T 9 min decreased and gradually shifted toward a longer retention time. The second and third peaks at R_T 10–15 min and R_T 15–20 min, respectively, continuously developed and showed little change after 8 h. The third peak finally reached the maximum intensity at 12 h of the reaction. This result was in agreement with a trend of the CA production previously reported (11,25). The third peak exhibited a broad fraction with a shoulder, indicating that multiple components might coexist with CA (Fig. 3, CA standard at R_T 16.5 min) in this fraction. Overall, the optimal reaction condition of isoamylase and TA α GT treatment for the highest CA production was that 8% (w/v) SPS of the initial starch substrate was debranched by isoamylase for 8 h and reacted by TA α GT for another 12 h at enzyme units and pH conditions explicated in the method section.

CA production by sequential treatment of isoamylase and TA α GT

CAs were produced under the optimum condition determined in the previous two sections. The yield of CA was estimated using HPSEC (7). The standard curve was prepared on the basis of HPSEC peak areas for different CA concentrations (0.1–1%, w/v). The resulting

calibration equation was $Y = 362030X + 6696.5$ ($R^2 = 0.9996$), where Y was assigned to a peak area of the CA product and X was assigned to a concentration of the CA product. As can be seen in Fig. 3A, a broader peak was observed for the CA products compared to the CA standard eluted at R_T 16.5 min, indicating a broader molecular weight distribution in the CA products. The maximum yield of CA from SPS was 48.56%. Notably, the CA production yield in this study is the highest level ever reported for the starches of normal amylose contents and is comparable to the maximum CA yield (47.25%) when using a high amylose starch (50–70% of amylose content) (11). In brief, the result stresses that the TA α GT treatment subsequent to isoamylase debranching is an effective procedure for a high yield of CA production using the SPS of normal amylose content.

Identification of CA products from SPS by sequential treatment of isoamylase and TA α GT

The cyclic forms of CA products were assessed via MALDI-TOFMS. The molecular masses of the cyclic and non-cyclic glucans were calculated as $162.14n \text{ Da} + 22.990 \text{ Da}$ (mass of Na^+ ion) and $162.14n \text{ Da} + 22.990 \text{ Da} + 18.015 \text{ Da}$ (mass of reducing end residue) respectively, where n is the number of monosaccharide (glucose) unit (26,27). It was confirmed that the final products were predominantly cyclic glucans with few linear molecules (Fig. 4A). The DP of CA products varied (DP 5–40), and their molecular weights

Fig. 4. The matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOFMS) analysis of (A) CA products formed by the sequential treatment of isoamylase and TA α GT and (B) reaction products formed by the simultaneous treatment of isoamylase and TA α GT.

agreed well with theoretical values for cyclic glucans. Although it was hypothesized that CA with a DP less than 6 is unlikely to exist due to steric overlaps when it cyclizes (28), a number of small cyclic molecules were observed from DP 5 to DP 22. This was attributed to higher proportions of A chains and short B chains in SPS amylopectin, relative to those in other normal starches (29). In addition, the small CA production with a minimum DP of 5 has been observed for the sequential treatment of isoamylase and α GT when using amylo maize starch as a substrate (11). Possibly, due to the difference in the amylose content and starch molecular structure of amylo maize starch and SPS, the DP profile of CA products from SPS was quite different from that from amylo maize starch (11), showing a greater portion at smaller DP (5–15). The physicochemical characteristics of CA products depending on the DP profile would be the focus of our future study.

CA production by simultaneous treatment of isoamylase and TA α GT CAs were produced from SPS by simultaneous addition of isoamylase and TA α GT, and their molecular weight distributions were assessed via HPSEC. As shown in Fig. 3B, the resulting peak at R_T 15–20 min appeared with a markedly low and broad pattern and the yield of CA production was as low as 3.31%. After additional debranching with isoamylase, the peak was sharpened and shifted to R_T 16.5 min, coincident with the CA standard (Fig. 3C). This result indicated that the multiple branched components, such as amylopectin clusters, cyclic cluster dextrans, and short-branched cyclic molecules, might exist. In addition, there were a number of linear α -1,4-glucans in final products, even greater than cyclic α -1,4-glucans as a result of MALDI-TOFMS (Fig. 4B). This can be attributed to the difference in the optimum reaction conditions of the two enzymes used in this study. In fact, both isoamylase and TA α GT attain only 60–80% of the

total activity under the given reaction condition for the simultaneous enzyme treatment. Screening or engineering a new debranching enzyme that possesses optimum reaction conditions comparable to those of TA α GT would be highly beneficial for the CA production from starch.

In summary, this study demonstrates that CA was successfully produced from SPS by sequential treatment with isoamylase (5 U/g, 8 h) and TA α GT (100 U/g, 12 h). The maximum yield was 48.56%, which is one of the highest levels ever reported for normal starches and is comparable to the yield from high-amylose starch. Utilizing normal starch with an average level of amylose content provides industrial potential in CA production owing to the low cost for the reaction substrate. The CA products varied in their molecular weights with a minimum DP of 5, despite the steric hindrance possibly involved in a cyclic form with DP 5. Thus, the conformational and physicochemical characterizations of those CA products might require further research. In contrast, the attempt to add both isoamylase and TA α GT simultaneously resulted in a low CA production yield (3.31%) with high proportions of branched molecules and linear α -1,4-glucans. Further efforts will need to explore a new line of isoamylase or α GT via bioengineering or genetic modification permitting one optimal reaction condition compatible with the other.

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Disclosure The authors declare no conflict of interest.

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