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Cycloamylose production from amylomaize by isoamylase and *Thermus aquaticus* 4-α-glucanotransferase

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ABSTRACT

Amylomaize (AMM) was utilized as the substrate for cycloamylose (CA) production in this study. After debranching, AMM was incubated with 10 U/g of *Thermus aquaticus* 4-α-glucanotransferase (TA 4αGTase) for various reaction times in water or in DMSO reaction system. The maximum conversion yield of CA was greatly improved from 24.55% to 45.58% and from 27.40% to 47.25% after debranching in the water and DMSO reaction systems, respectively. Compared with the method that produced CA from commercial potato amylose, this method produced CA from a natural amylopectin containing starch with enhanced conversion yield after debranching. Meanwhile, we found that the minimum degree of polymerization (DP) of CA from TA 4αGTase treatment was 5, regardless of the reaction conditions. These results were different from those reported in the literature that stated the minimum DP of CA produced with a TA 4αGTase treatment was 22 regardless of the reaction conditions.

1. Introduction

4-α-Glucanotransferase (EC 2.4.1.25) (4αGTase), which belongs to the α-amylase super family, was first found in *Escherichia coli* and later reported in many bacterial species (Terada, Fujii, Takaha, & Okada, 1999). The 4αGTase can catalyze the transfer of one α-1,4-glucan to another α-1,4-glucan or to glucose. This intermolecular glucan transfer reaction is readily reversible, and is often called disproportionation. Meanwhile, this enzyme can catalyze intramolecular glucan transfer reactions, which produce cycloamylose (CA) (Takaha & Smith, 1999; Takaha, Yanase, Takata, Okada, & Smith, 1998). This reaction is also reversible and often referred as a coupling reaction.

CA is a cyclic molecule consisting of α-1,4-linked glucose units. It has a broad degree of polymerization (DP) distribution that ranges from nine to a few hundred (Takaha, Yanase, Takata, Okada, & Smith, 1996). The minimum DP of CA is variable based on the source of the enzyme. For instance, the 4αGTase from *Thermus aquaticus* produces CA_{22} as the smallest CA, while the 4αGTases from *E. coli* and potato both produce CA_{17} as the smallest CA (Takaha & Smith, 1999). CA possesses a high water-solubility and a relatively large hydrophobic cavity, thus, it is likely able to protect vulnerable guest molecules through inclusion complexation by changing the solubility, reactivity, and stability of guest molecules (Kim et al., 2011). It was reported that CA can be used as a biomaterial in a gene delivery system (Toita, Morimoto, & Akiyoshi, 2010). Also, CA can be used as a nanogel for siRNA delivery (Toita, Soma, Morimoto, & Akiyoshi, 2009). In a protein refolding system, CA acted as an efficient artificial chaperone, assisting to prevent protein aggregation and enhance the degree of protein refolding (Machida et al., 2000). Moreover, interaction between CA and various drugs can greatly improve the solubility and bioavailability of the drugs (Baek et al., 2011, 2012; Tomono et al., 2002). Generally, the commercial potato amylose was supposed to be the best substrate for CA production (Kim et al., 2011). However, because the preparing of commercial potato amylose was difficult, complex and time-consuming, mass production and wide application of CA were considerably restricted.

Abbreviations: CA, cycloamylose; TA 4αGTase, *Thermus aquaticus* 4-α-glucanotransferase; DP, degree of polymerization; AMM, amylomaize.

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In this paper, we utilized amylomaize (AMM) as the substrate for CA preparation, for AMM is rich in amylose and its amylopectin has long side-chains, and a sequential dual enzymatic method was introduced. First, AMM was debranched by isoamylase. Then it was utilized as a substrate for CA production by TA 4αGTase. In addition, to investigate the effect of debranched AMM amylopectin on CA production, amylose and amylopectin were separated from AMM and used as the substrates, respectively. The key objective of this study was to produce CA by sufficiently utilizing the amylopection in a natural starch and to improve the conversion yield of CA from a natural starch.

2. Materials and methods

2.1. Materials

Potato amylose was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Amylomaize V was purchased from Puluoxing Starch Co. (Hangzhou, China). Amyloglucosidase from *Rhizopus* sp. and isoamylase from *Pseudomonas* sp. were purchased from Megazyme International Ireland Ltd. (Bray, Ireland). Other chemicals were analytical grade.

2.2. Preparation of TA 4αGTase

Gene cloning and transformation of TA 4αGTase were performed according to Park et al. (2007). The *E. coli* transformants harboring TA 4αGTase gene were grown on Luria–Bertani media (1% Bacto-tryptone, 0.5% yeast extract, 1% NaCl) at 37 °C with ampicillin (100 μg/ml) for 10 h for enzyme production. The 6× His-tagged target protein was purified using Ni-NTA affinity column and the purity of the protein was confirmed by sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE). The optimum temperature and pH for TA 4αGTase were determined as previously described (Terada et al., 1999).

2.3. Enzyme activity

TA 4αGTase activity was determined by measuring the optical change in iodine-staining during the conversion of amylose by the enzyme (Liebl, Feil, Gabelsberger, Kellermann, & Schleifer, 1992). The reaction mixture contained 250 μl of 0.2% potato amylose (w/v), 50 μl of 1% maltose (w/v), 600 μl of 50 mM Tris–HCl buffer (pH 7.5), and 100 μl of the purified enzyme solution and it was incubated at 70 °C for 10 min. Then the mixture was boiled for 10 min to inactivate the enzyme. Aliquots (100 μl) were mixed with 1 ml of iodine solution (0.02% I_2 + 0.2% KI), and the absorbance at 620 nm was measured immediately using an UV/Vis spectrophotometer (Mapada V-1800, Shanghai, China). One unit of TA 4αGTase was defined as the amount of enzyme that degrades 0.5 mg/ml of potato amylose per min under the assay conditions used. The protein concentration was determined using the Bradford (1976) method with bovine serum albumin as a standard.

2.4. Quantification of CA by HPSEC

The synthesis of CA standard from potato amylose by TA 4αGTase was performed as previously described (Kim et al., 2011). The standard curve of CA was fitted using the pure CA standard with high-pressure size-exclusion chromatography (HPSEC). Pure CA standard (0.01–0.2%) was dissolved in distilled water and filtered through a 0.45 μm syringe filter. Then, the standard was separated with Shodex OHpak SB-804 HQ and OHpak SB-802 HQ columns (Showa Denko, Tokyo, Japan) in tandem and the standard was analyzed by a Hitachi HPLC system (Hitachi, Tokyo, Japan) combined with a refractive index detector (Hitachi RI-5450, Hitachi, Tokyo, Japan). The columns were maintained at 50 °C and HPLC grade water was used as an eluent at a flow rate of 0.8 ml/min. Glucans (Sigma–Aldrich, St. Louis, MO) with molecular masses of 1000 Da, 5000 Da, 12,000 Da, 150,000 Da and 670,000 Da were used as molecular mass standards for the molecular weight calibration curve. On the basis of the peak area of each CA concentration, the standard curve was prepared. The conversion yields of CA products from various substrates in this study were quantified using the standard curve.

2.5. Synthesis of CA from AMM

AMM was debranched using the method described by Han, BeMiller, Hamaker, & Lim (2003). The debranched AMM (1%, w/v) was mixed with 6 volumes of ethanol (99.9%), followed by centrifugation at 5000 × *g* for 10 min. The precipitates were dissolved in 1 ml of preheated 50 mM Tris–HCl buffer (pH 7.5) and then boiled for 30 min. This substrate solution was cooled and 10 U/g of TA 4αGTase was added. After enzyme inactivation, the reaction mixture was diluted in 4 ml of 50 mM sodium acetate buffer (pH 5.5) combined with incubation with 0.4 U/ml of amyloglucosidase at 50 °C for 6 h, then the resulting solution was boiled for 10 min to inactivate the enzyme. The denatured proteins were removed by centrifugation at 10,000 × *g* for 10 min and the supernatant was mixed with 10 volumes of ethanol (99.9%). Afterwards, the amyloglucosidase-resistant CA products were collected by centrifugation (10,000 × *g*, 10 min) at 25 °C and washed twice, first with 10 volumes of 85% ethanol and then with 10 volumes of 80% ethanol. After completely drying in an oven at 40 °C for 12 h, various CA products were obtained. The conversion yields of CA products based on the initial amount of AMM were calculated from the peak areas of HPSEC analysis using the standard curve as described in Section 2.4.

2.6. Synthesis of CA from AMM amylose and AMM amylopectin

Amylose and amylopectin from AMM were obtained according to the procedure of Krishnaswamy and Sreenivasan (1948). Afterwards, AMM amylopectin was debranched under the same conditions as AMM, then 1 ml of 1% (w/v) AMM amylose, AMM amylopectin and debranched AMM amylopectin were reacted with 10 U/g of TA 4αGTase under the optimum conditions. The CA products were obtained by following the procedure described in Section 2.5 and the conversion yields of CA from AMM amylose, AMM amylopectin and debranched AMM amylopectin were calculated at the same time intervals as other substrates in this study. Molecular weight distributions of AMM amylose and debranched AMM amylopectin were analyzed by HPSEC as described in Section 2.4 with a flow rate of 1.0 ml/min. The peak DPs of AMM amylose and debranched AMM amylopectin were calculated directly from the molecular weight standard curve and their average DPs were calculated as described by Potocki-Veronese et al. (2005).

2.7. Analysis of CA by MALDI-TOF MS

The molecular-mass spectrum of pure CA was collected using a matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS; New ultrafleXtreme MALDI TOF/TOF, Bruker Daltonics Inc., Billerica, USA). The purified CA was dissolved in distilled water (10 mg/ml). 2,5-Dihydroxybenzoic acid (2,5-DHB) was prepared as 1% (w/v) solution and was used as a matrix. The sample solution and matrix were mixed in equal volumes (1.0 μl each), and they were spotted on a sample plate followed by air-drying until homogeneous crystals formed. The dried sample on the plate was then applied to the mass spectrometer and analyzed with an accelerated voltage and reflective voltage of 19 kV

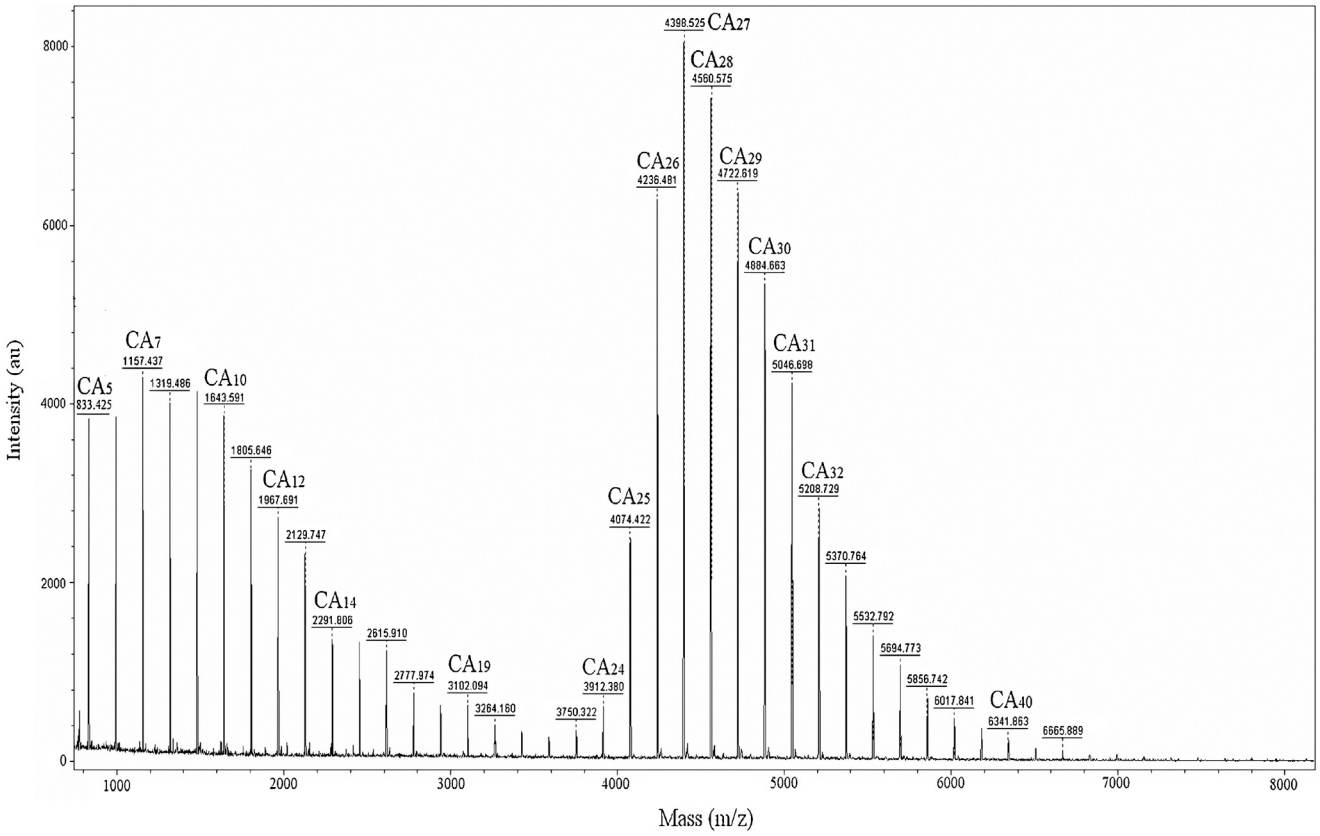

Fig. 1. MALDI-TOF MS analysis of CA produced from commercial potato amylose. The subscript "*n*" on CA_n indicates the DP of CA. The number above each peak indicates the molecular mass (in Daltons) of the molecule plus 23 Da (sodium ion).

and 16 kV, respectively. The molecular mass of CA was detected in the range of 600 to 10,000 Da in reflection mode.

2.8. Analysis of CA by HPAEC

The purified CA in this study was analyzed by high performance anion exchange chromatography with a pulsed amperometric detector (HPAEC-PAD, Dionex-300, USA). A CarboPac PA-100 column (240 × 4 mm, Dionex, USA) was used and the sample was eluted with a linear gradient of 600 mM sodium acetate from 0 to 100% over 70 min in 150 mM NaOH at a flow rate of 1 ml/min (Park et al., 2007). α-Cyclodextrin, β-cyclodextrin and γ-cyclodextrin were used as standards and analyzed under the same HPAEC conditions. Each purified CA products was firstly analyzed by MALDI-TOF MS and then analyzed by HPAEC.

2.9. Statistical analysis

All numerical results in this study are averages of three independent replicates. Data were analyzed by one-way analysis of variance (ANOVA) using ORIGIN 8.5 (OriginLab Inc., USA). The means comparisons were determined by Tukey's test ($P < 0.05$).

3. Results and discussion

3.1. Synthesis of CA standard from potato amylose

To quantify CA products from various reaction conditions, the CA standard was obtained by using commercial potato amylose as a substrate for TA 4αGTase treatment. The purity and structure of CA standard were evaluated by MALDI-TOF MS (Fig. 1), and it was confirmed that no linear glucans remained in the CA standard. On the MALDI-TOF MS spectrum, the molecular masses and DPs of the CA standard were indicated on the peaks, among which the intensity of CA_{27} (Mw of $[CA_{27}+Na]^+$ = 4398 Da) was greatest and the minimum DP of the CA standard was 5. After determination by MALDI-TOF MS, the DP distribution of the CA standard was confirmed further by HPAEC. Under HPAEC analysis, α-cyclodextrin, β-cyclodextrin and γ-cyclodextrin with DP 6, 7 and 8 were eluted at 5.5 min, 7.4 min and 9.0 min, respectively. For CA standard, a peak appeared before 5.5 min. Together with the results obtained from MALDI-TOF MS, the DP of this peak was determined as 5. And the DP distribution of the CA standard analyzed by HPAEC was coincident with the result from MALDI-TOF MS (Fig. 2).

A HPSEC standard curve was generated using the highly pure CA standard to quantify CA products from various enzyme reaction conditions in this study (Fig. 3). On the HPSEC chromatogram, the apex of each CA peak was constantly eluted at about 18.5 min and each different concentrations of CA standard had different peak areas. Different concentrations and peak areas of the CA standard were prepared to the standard curve. The peak area of the CA standard mixture was linearly correlated with its amount in a perfect manner from 0.01% to 0.2% (w/v; $R^2 = 0.999$).

3.2. Synthesis of CA from AMM

The synthesis of CA products from AMM was conducted by combined treatment of isoamylase and TA 4αGTase in a sequential order. Amyloglucosidase was used to hydrolyze the residual linear α-(1,4)-linked glucans as a necessary step for purification of CA products. The optimum dosage of TA 4αGTase was determined by using commercial potato amylose, AMM and debranched AMM as substrates for CA production up to 6 h; the maximum conversion yields were achieved when the dosage of TA 4αGTase was

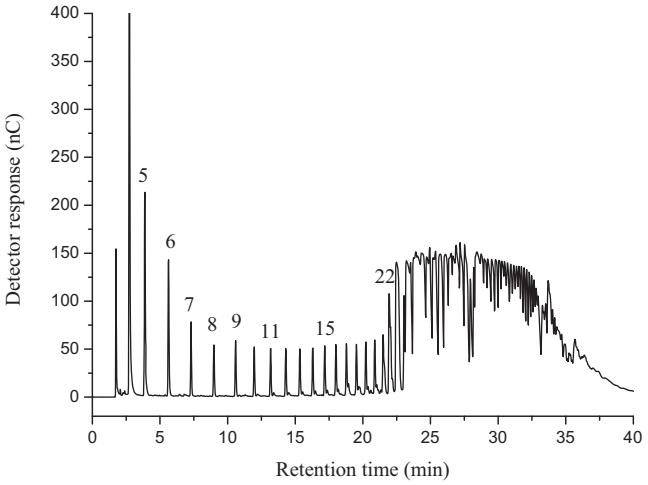

Fig. 2. HPAEC analysis of CA standard produced from commercial potato amylose. The DPs of smaller CA molecules were indicated on the peaks.

10 U/g regardless of the substrates (data not shown). Under the optimum reaction conditions, the conversion yields of CA products from potato amylose, AMM and debranched AMM at different reaction times were calculated (Fig. 4).

In the initial reaction time, the conversion yields of CA from the three substrates increased rapidly and the maximum conversion yields were achieved after 12 h of reaction, after that, all the conversion yields decreased. It has been reported that 4αGTase may hydrolyze its own CA products (Fujii et al., 2005; Przylas et al., 2000), resulting in a decrease of conversion yield. On the other hand, as the reaction proceeded, low molecular weight sugars increased, which facilitated the coupling reaction to form linear glucans, leading to a decrease of CA products as well. The maximum conversion yield of CA from AMM was 24.55% in water reaction system, whereas after debranching, it can reach up to 45.58%, increasing 1.9 folds.

In our work, the conversion yields of CA in the reaction system that contained 10% DMSO were also calculated (Fig. 4 B) and the maximum conversion yield was achieved after 12 h of reaction. In the DMSO reaction system, the maximum conversion yield of CA from AMM was 27.40%, whereas after debranching, it can reach up to 47.25%. The conversion yields of CA in the DMSO reaction system were generally higher than those in the water reaction system no matter what the substrates were. This result was partially explained

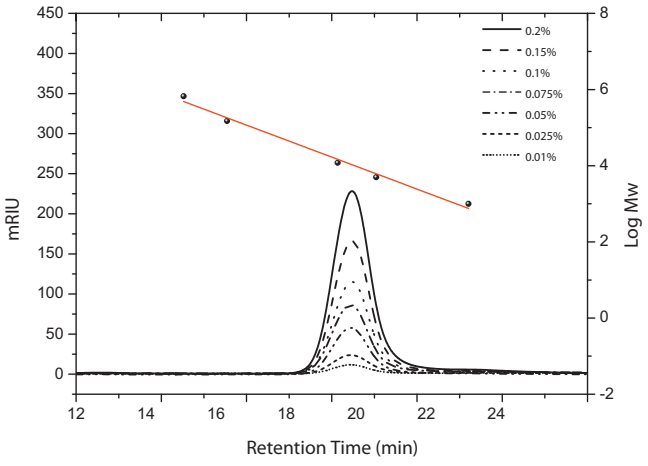

Fig. 3. HPSEC analysis of the CA standard mixture (0.01–0.2%) produced from commercial potato amylose by TA 4αGTase treatment.

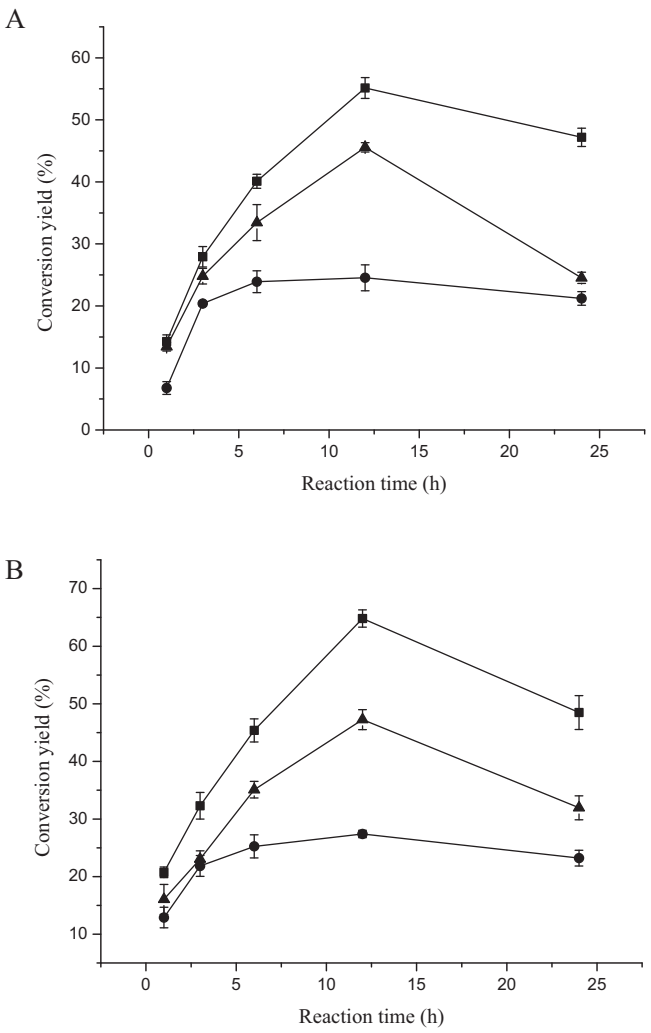

Fig. 4. Conversion yields of CA products with potato amylose, AMM, debranched AMM as substrates in water reaction system (A) and DMSO reaction system (B). The symbols in the graph indicate different substrates: (■) potato amylose; (●) AMM; (▲) debranched AMM.

by the increase in the solubility of linear glucans as the addition of DMSO. In addition, compared with the maximum conversion yield of CA from debranched AMM in the DMSO reaction system, the maximum conversion yield of CA from debranched AMM in the water reaction system was just a little lower (1.67%). So, the DMSO reaction system can be replaced by the water reaction system when combined treatment of isoamylase and TA 4αGTase was utilized to produce CAs in our study.

3.3. Synthesis of CA from AMM amylose and AMM amylopectin

In order to investigate the effect of α-(1,4)-linked glucans produced from debranched AMM amylopectin on CA production, amylose and amylopectin from AMM were obtained. Molecular weight distributions of AMM amylose and debranched AMM amylopectin were analyzed by HPSEC (Fig. 5). On the HPSEC chromatogram, AMM amylose had a wide molecular weight distribution with its DP ranging from 24,684 to 16, while debranched AMM amylopectin had a narrow molecular weight distribution with its DP ranging from 457 to 6. Their average DPs were calculated as 592 and 29, respectively (Fig. 5).

Under the optimum conditions, AMM amylose, AMM amylopectin and debranched AMM amylopectin were reacted with

Table 1
The conversion yields of CA products from AMM, debranched AMM, AMM amylose, AMM amylopectin and debranched AMM amylopectin at different reaction times.[a]

	Conversion yield/%				
Time/h	1	3	6	12	24
AMM	$6.77^{b,C}$	$20.39^{c,B}$	$23.91^{b,AB}$	$24.55^{b,A}$	$21.21^{c,AB}$
debranched AMM	$13.41^{a,D}$	$24.80^{b,C}$	$33.43^{a,B}$	$45.58^{a,A}$	$24.53^{b,C}$
AMM amylose	$13.03^{a,D}$	$29.42^{a,C}$	$35.02^{a,B}$	$49.31^{a,A}$	$34.64^{a,B}$
AMM amylopectin	$0.39^{c,C}$	$4.69^{e,B}$	$8.96^{d,A}$	$7.78^{d,A}$	$5.13^{e,B}$
debranched AMM amylopectin	$12.40^{a,B}$	$14.36^{d,A}$	$12.66^{c,AB}$	$12.24^{c,B}$	$12.25^{d,B}$

Values followed by different uppercase superscripts in each line and different lowercase superscripts in each column are significantly different ($P < 0.05$).
[a] Means of triplicates.

10 U/g of TA 4αGTase in water reaction system and the conversion yields were calculated at the same time intervals as AMM and debranched AMM. Table 1 summarizes the conversion yields of CA products from AMM, debranched AMM, AMM amylose, AMM amylopectin and debranched AMM amylopectin. Both debranched AMM and AMM amylose reached their maximum conversion yields after 12 h of reaction, whereas debranched AMM amylopectin reached its maximum conversion yield after 3 h of reaction and as the reaction proceeded, the conversion yields decreased slowly. This may be because of the shorter side-chain lengths of debranched AMM amylopectin compared with AMM amylose. It was reported that the size of linear glucans may be one of the important factors in determining the conversion yield of CA (Endo, Zheng, & Zimmermann, 2002), so the conversion yields of CA from debranched AMM amylopectin were lower than those from AMM amylose at any reaction times. The conversion yields of CA from AMM amylopectin were always lower than those from debranched AMM amylopectin (significantly different), indicating the debranched AMM amylopectin was a more suitable substrate for CA production than AMM amylopectin. Compared with the conversion yields of CA from AMM, the conversion yields of CA from debranched AMM were higher than those from AMM at any reaction times. Even after 24 h of reaction, the conversion yields of CA from AMM and debranched AMM were significantly different at the 0.05 level. Especially, the conversion yields of CA from debranched AMM could reach to those from AMM amylose when the reaction time was around 6 h to 12 h (not significantly different). The reason for the extremely high CA yield from debranched AMM can be contributed to the stimulative effect of α-(1,4)-linked glucans from debranched AMM amylopectin on CA production during that period of reaction time (6–12 h).

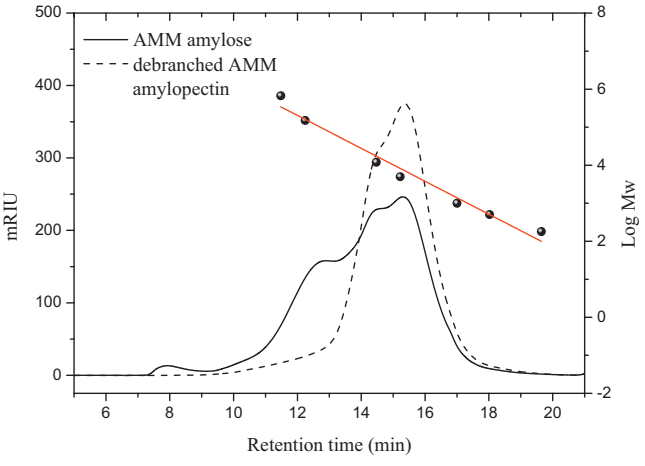

Fig. 5. Molecular weight distributions of AMM amylose and debranched AMM amylopectin analyzed by HPSEC.

3.4. Comparison of CA production from debranched AMM and potato amylose

In water reaction system, the maximum conversion yield of CA from commercial potato amylose was 55.15% under the optimum conditions while the maximum conversion yield of CA from debranched AMM was 45.58%. Although the conversion yield of CA from potato amylose was the highest among the substrates utilized in this work, it was difficult and complex to prepare. The amylose preparing process involved at least three cycles of crystallization with n-butyl alcohol, which was time-consuming and environmentally unfriendly. On the other hand, when AMM was debranched and used as a substrate for CA production, the maximum conversion yield of CA products can reach up to 45.58% in water reaction system, which was 1.9-fold more than AMM without debranching, indicating the importance of the starch debranching method for CA production. Because of the easy accessibility of native starch, production of CA from AMM by combined treatment of isoamylase and TA 4αGTase showed great potential for use on an industrial scale.

3.5. The minimum DP of CA from TA 4αGTase treatment

It is generally considered that TA 4αGTase produces CA with a minimum DP of 22 (Terada et al., 1999), but interestingly, in our work, we found that the minimum DP of CA from TA 4αGTase treatment was 5. Cho et al. (2009) mentioned similar results without further discussion. In order to confirm this result in our work, CA products were detected by MALDI-TOF MS in linear and reflection mode separately, but the results were always the same. As the reflection mode has a higher resolution, CA products from various reaction conditions were detected in reflection mode in further experiments. In this study, MALDI-TOF MS was applied to detect CA as it is a suitable tool for the analysis of saccharides, and it has been reported that MALDI-TOF MS can provide the molecular masses of compounds with carbohydrate moieties (Harvey, 1996, 1999; Rouse & Vath, 1996).

The structures of CA products from debranched AMM at different reaction times were analyzed by MALDI-TOF MS (Fig. 6). And it was also suggested that the minimum DP of CA products was 5. In addition, the CAs produced from AMM amylose and debranched AMM amylopectin at different reaction times exhibited the same minimum DPs as potato amylose and debranched AMM (Fig. 7). The results indicated that the minimum DP of CA from TA 4αGTase treatment was 5 regardless of the reaction conditions. According to the DP distribution of CA from the results of MALDI-TOF MS, CA can be separated into two parts: one with smaller CA molecules that had DPs below 22 and the other with larger CA molecules that had DPs above 22. During the initial reaction time, the amount of smaller CA molecules (DP < 22) can account for nearly 50% of all CA molecules, while when the reaction time reached to 24 h, the smaller CA molecules reduced (Figs. 6 and 7) and larger CA molecules (DP > 22) became the main constituents. It was reported that cyclodextrin glucanotransferase (CGTase) and D-enzyme preferentially formed larger cyclic glucans in the initial reaction stage

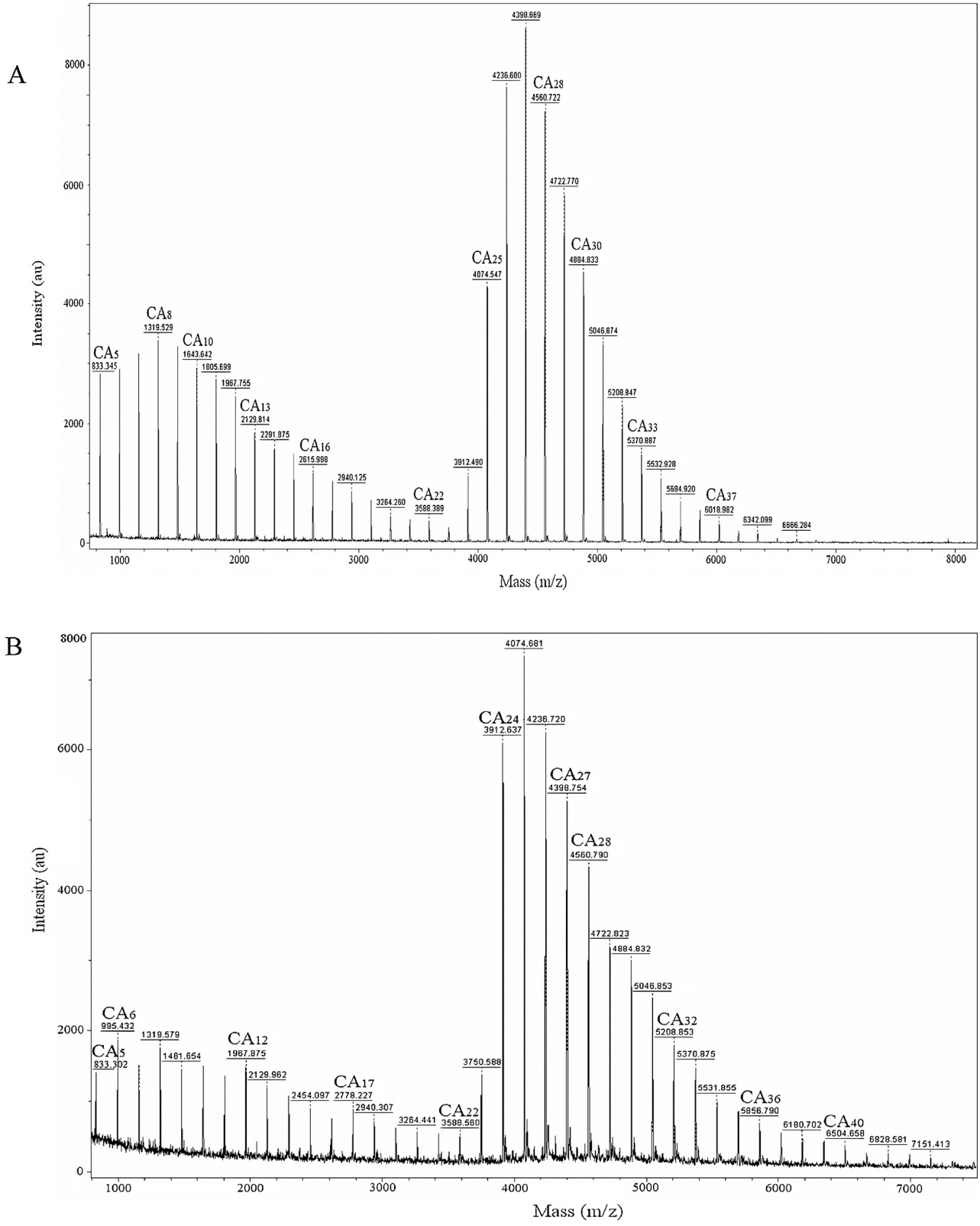

Fig. 6. MALDI-TOF MS analysis of CA products from debranched AMM at different reaction times: 1 h (A) and 24 h (B).

and, subsequently, smaller cyclic glucans (Takaha et al., 1996; Terada, Yanase, Takata, Takaha, & Okada, 1997), but in our study, this phenomenon was not observed in TA 4αGTase. Although TA 4αGTase and D-enzyme were similar enzymes, they were different in this enzymatic property. For D-enzyme, average Mw of cyclic glucans it produced decreased from ~70,000 to ~10,000 over reaction time and this decrease occurred during the very beginning of the reaction stage (<1 h). While for TA 4αGTase, smaller and larger cyclic glucans emerged together throughout the reaction, and the smaller CA molecules decreased after 1 h of reaction. The reduced

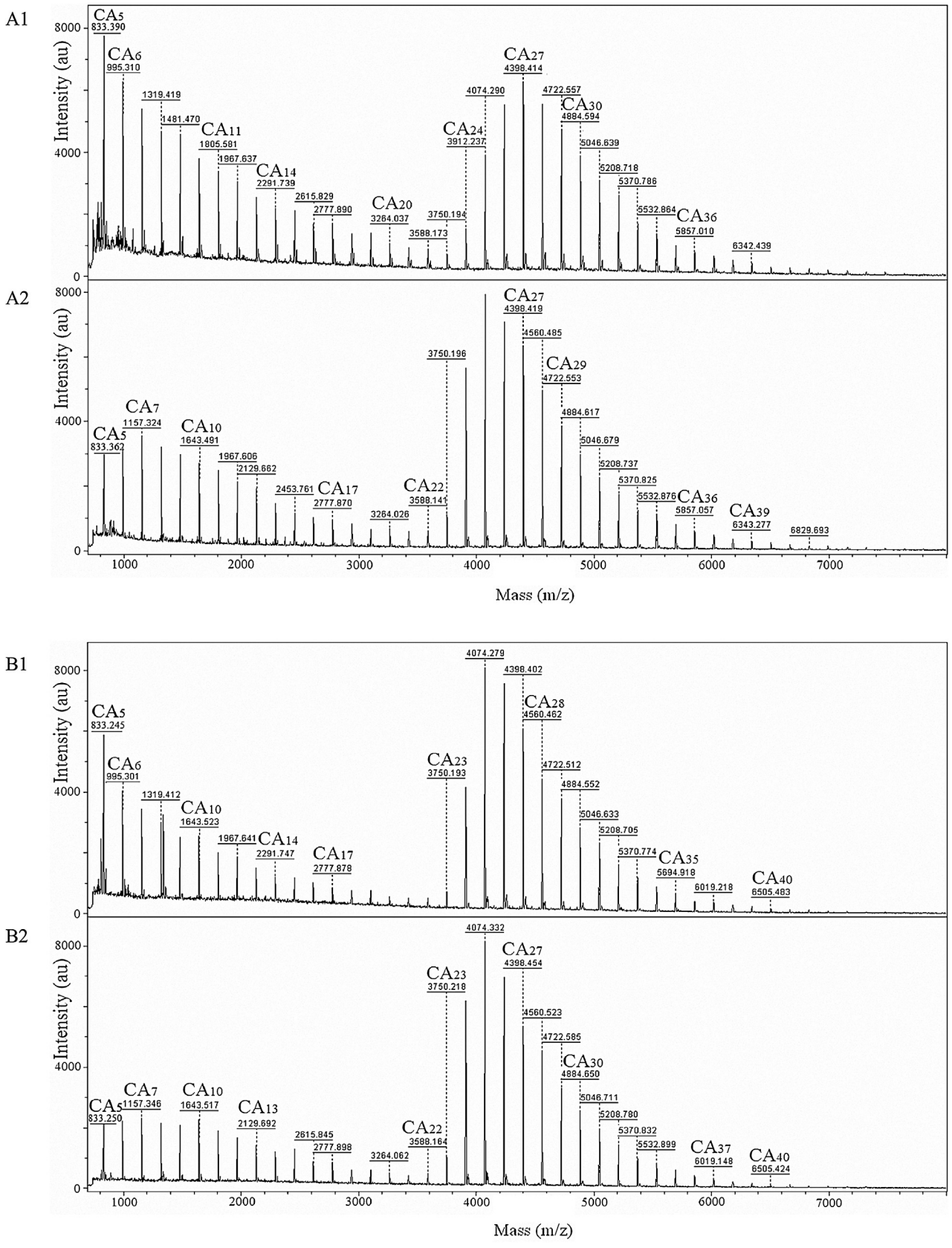

Fig. 7. MALDI-TOF MS analysis of CA products from AMM amylose (A) and debranched AMM amylopectin (B) at different reaction times: 1 h (A1, B1), 24 h (A2, B2).

smaller cyclic molecules may result in the formation of larger cyclic molecules by two sequential reactions: (1) a coupling reaction that forms larger linear molecules and (2) a cyclization reaction that forms larger CA molecules. This sequence remains to be confirmed in further experiments.

4. Conclusions

For the first time, we reported a bioconversion process of CA production by the combined treatment of isoamylase and TA 4αGTase. This process involved a usage of natural starch substrate which usually contains large amount of amylopectin, and a higher conversion yield of CA products was achieved after debranching. Compared with the amylopectin in AMM, the debranched AMM amylopectin was a more suitable substrate for CA production. And the glucans from debranched amylopectin had a stimulative effect on CA production when the reaction time was 6–12 h. Thus, the maximum conversion yield of CA products from AMM was 24.55%, while after debranching, it reached to 45.58% in water reaction system. In contrast to previous reports, we found that the minimum DP of CA from TA 4αGTase treatment was 5 regardless of the reaction conditions and as the reaction proceeded, the amount of smaller cyclic glucans decreased. In further study, starches from different sources can be utilized to produce CA through combined treatment of isoamylase and TA 4αGTase. In addition, other 4αGTases from various bacterial species can be used to produce CA from natural starches by the method we reported as well.

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