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One-Pot Synthesis of Cycloamyloses from Sucrose by Dual Enzyme Treatment: Combined Reaction of Amylosucrase and 4-α-Glucanotransferase

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ABSTRACT: Amylose-like α-(1,4)-glucan known as the most favorable substrate for the enzymatic production of cycloamyloses (CAs) using 4-α-glucanotransferase has a solubility issue, which requires an additional solubilization process. In our study, two glucosyltransferases, *Synechocystis* 4-α-glucanotransferase and *Neisseria* amylosucrase, were adopted to develop an efficient biocatalytic production process of CAs directly from sucrose. From one-pot synthesis, the maximum CA yield (9.6%, w/w) with 0.3 M sucrose was achieved with 10 units/mL of amylosucrase and 0.1 unit/mL of 4-α-glucanotransferase at 40 °C for a 3 h reaction in a simultaneous dual enzyme reaction mode. The size of linear α-(1,4)-glucan was positively related to the CA productivity by 4-α-glucanotransferase in a hyperbolic manner. Using our innovative bioprocess, there was no practical limitation on the initial sucrose concentration and no use of insoluble linear α-(1,4)-glucan substrate. Consequently, the concomitant dual enzyme reaction converted sucrose directly to CAs via *in situ* transient linear α-(1,4)-glucan as an soluble intermediate.

KEYWORDS: Cycloamylose, amylosucrase, 4-α-glucanotransferase, one-pot synthesis

INTRODUCTION

Cycloamyloses (CAs), which are macrocyclic glucans consisting of α-(1,4)-linked glucose units, may form hydrophobic channels, such as the V-type amylose helix structure.[1] CAs show high solubility in aqueous solution,[2] and the V-type helical conformation can form inclusion complexes with various hydrophobic guest molecules.[3] Thus, CAs should be able to protect vulnerable guest compounds through inclusion complexation by changing their solubility, reactivity, and stability. The similarity of CAs in inclusion-complexing capability to cyclodextrins (CDs) can find a wide range of technical applications in food, cosmetics, and pharmaceutical industries. Unlike CDs, studies on the CA application have been pursued in very limited area. Among the few studies, the interactions between the mixtures of CAs with degree of polymerizations (DPs) of 20−55 and drugs, such as prednisolone, cholesterol, digoxin, digitoxin, and nitroglycerin, have been targeted for a potential application of CAs.[3] In addition, CAs provided an efficient method for refolding denatured antibody to correct its active structures.[4] In a couple of studies, CAs have been chemically modified to improve their functionality. CAs were conjugated to a sulfur-containing small chemical to enhance its chemiluminescence efficiency by forming a CA-bound luciferin analogue.[5] Recently, cationized CAs was synthesized by introducing spermine groups and used as a gene-delivery system.[6]

4-α-Glucanotransferase (4αGTase) (EC 2.4.1.25) is one of the enzymes that relocates α-(1,4)-linkages in glucans and uniquely produces CAs by intramolecular cyclization of α-(1,4)-glucans. Cyclodextrin glucanotransferase (EC 2.4.1.19) and amylomaltase in bacterial species, as well as disproportionating enzyme in plants, belong to this enzyme group.[7−9] Dependent upon the source of the enzyme, the minimum DP of CAs was from 16 to 22[8,10,11] and the maximum DP reaches several hundreds.[9]

Presumably, α-(1,4)-linked linear glucans were supposed to be the best substrate for CA production because of the reaction specificity of 4αGTase. Thus, synthetic amylose, which is essentially linear α-(1,4)-glucan, was widely used to characterize this type of enzyme in terms of cycloamylose production. Until now, several enzymatic synthesis methods of amylose have been reported. Glucan phosphorylase (EC 2.4.1.1) can produce amylose from its substrate, glucose-1-phosphate (G-1-P), but this phosphorylated glucose is too expensive for industrial applications.[12,13] Originally, amylose is synthesized by starch synthase (EC 2.4.1.11) in the plant system, and this enzyme uses ADP−glucose as a substrate. However, this activated sugar is not readily available for mass production of amylose. It is most promising to find a way to provide G-1-P efficiently for amylose production. A possible solution is to combine an enzyme that can supply G-1-P. Sucrose phosphorylase (EC 2.4.1.7) and cellobiose phosphorylase (EC 2.4.1.20) can convert sucrose and cellobiose to a common product, G-1-P, respectively. Thus, a combined reaction system of two phosphorylases can lead to amylose synthesis. Besides these possible approaches, a unique amylosucrase (EC 2.4.1.4) was reported and successfully synthesized amylose from sucrose without any primer molecule.[14,15]

Previously, we cloned and expressed recombinant 4αGTase from *Synechocystis* sp. PCC 6803 and fully characterized its biochemical properties.[11] In this study, *Synechocystis* 4αGTase

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was used for CA bioconversion, and the other glucosyltransferase, which is amylosucrase from *Neisseria polysaccharea* (NpAS), was adopted for supplying amylose-like linear α-(1,4)-glucan substrate to improve the biocatalytic process of CA production. Synthetic amylose *in vitro* is not readily soluble and crystallized into various forms. Thus, amylose substrate should be dissolved in either aqueous dimethyl sulfoxide with heating or strong alkaline solution for enzymatic CA production.

The key objective of this study was to investigate dual enzyme reaction conditions for optimizing the CA production process. For the first time, without preparing synthetic amylose substrate, one-pot synthesis of CAs was attempted directly through a coordinated bioconversion of highly soluble sucrose by two key enzymes, NpAS and 4αGTase.

MATERIALS AND METHODS

Materials. Potato amylose and sucrose were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Glucoamylase from *Rhizopus* sp. was obtained from Toyobo Co. (Osaka, Japan). Pullulan Shodex standard P-82 (P5, $M_w = 5.80 \times 10^3$ g/mol; P10, $M_w = 1.18 \times 10^4$ g/mol; P20, $M_w = 2.28 \times 10^4$ g/mol; P50, $M_w = 4.73 \times 10^4$ g/mol; P100, $M_w = 1.12 \times 10^5$ g/mol; and P200, $M_w = 2.12 \times 10^5$ g/mol) was purchased from Showa Denko Co. (Tokyo, Japan).

4αGTase Enzyme Assay. The recombinant 4αGTase of *Synechocystis* sp. was cloned and expressed as previously described.[11] In the presence of maltose, the observed change in iodine-absorbing properties during the conversion of amylose by 4αGTase was used as a basis for this assay. The assay mixture of 0.05% (w/v) amylose, 0.05% (w/v) maltose, 50 mM phosphate (pH 7.0) buffer, and the enzyme was incubated at 45 °C. An aliquot (0.1 mL) of the reaction mixture withdrawn at 0 and 10 min was mixed with 1 mL of 0.02% iodine/potassium iodide solution (Lugol's solution), and the absorbance at 620 nm was measured immediately with a spectrophotometer (DU 730, Beckman Coulter). The absorbance of the 10 min incubated sample was subtracted from the value of the corresponding zero-time sample. A total of 1 unit of 4αGTase activity was arbitrarily defined as the amount of enzyme that causes a change in absorbance by 1 unit in 10 min under the reaction conditions. The assay was reproducible, and the absorbance change was linear for the applied amount of enzyme.[16]

NpAS Enzyme Assay. The recombinant NpAS was cloned and expressed as reported earlier.[17,18] A total of 1 unit of NpAS was defined as the amount of enzyme that releases 1 μmol of fructose/min. The enzyme assay was carried out in 50 mM Tris-HCl buffer (pH 7.0) containing 0.1 M sucrose and 0.1% (w/v) waxy corn starch at 35 °C for 10 min. After the reaction, the amount of released fructose was measured by the dinitrosalicylic acid method, using fructose as a standard.[19]

CA Production from Potato Amylose by 4αGTase. A total of 10 mg of potato amylose (1%, w/v) was completely wetted with 0.1 mL of water for 10 min and then dispersed in 0.9 mL of dimethyl sulfoxide (DMSO). The dispersion in a glass vial was boiled in a water bath for 1 h with constant stirring and additionally stirred for 12 h at room temperature to obtain a clear solution. The resulting solution was mixed with 6 volumes of ethanol (99.9%), followed by centrifugation at 4500*g* for 10 min. The precipitates were dissolved in 1 mL of preheated 50 mM sodium phosphate buffer (pH 7.0) and then boiled for 30 min.[20] This substrate solution of potato amylose was cooled and treated with 0.1 unit/mL of 4αGTase at 45 °C for 2 h in a shaking water bath. After the incubation, the reaction mixture was heat-treated in boiling water for 10 min to inactivate the enzyme.

Purification of the CA Mixture Product as a Quantification Standard. A total of 1 mL of the 4αGTase reaction mixture was suspended in 4.0 mL of 50 mM sodium acetate buffer (pH 5.5) and incubated with 0.4 unit/mL of glucoamylase at 50 °C for 6 h to completely hydrolyze the residual linear glucans to glucose.[7,9] The reaction was completed by heating the mixture for 10 min. It was then centrifuged at 9000*g* for 10 min at 25 °C to remove the denatured proteins. The supernatant was mixed with 10 volumes of ethanol (99.9%), and then the glucoamylase-resistant CAs were recovered as a form of precipitates by centrifugation (9000*g*, 10 min). The collected CAs were washed twice with 10 volumes of 85 and 80% ethanol in this order to completely remove the released glucose from glucoamylase treatment. The residual ethanol in the finally centrifuged CAs was evaporated by heating for 10 min in a boiling water bath. The highly purified CAs were obtained after completely drying CAs in an oven at 50 °C for 12 h, and this CA mixture was used for the quantification of CA products obtained from various 4αGTase reaction conditions in this study.

Purity and Structure Analysis of the CA Product by Matrix-Assisted Laser Desorption/Ionization−Time-of-Flight Mass Spectrometry (MALDI−TOF MS). The molecular-mass spectrum of pure CAs was collected by MALDI−TOF MS. The purified CAs were dissolved in distilled water (0.5 mg/10 μL). 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 the plate. After air-drying the spotted sample mixture, the molecular-mass profile of CAs was analyzed by MALDI−TOF MS. The CalMix2 was used as a calibration standard.[21]

Standard Curve Fitting and Quantification of CAs by High-Pressure Size-Exclusion Chromatography (HPSEC). The standard curve of CAs was fitted using the pure CAs by HPSEC (Summit HPLC System, Dionex, CA) combined with a refractive index detector (Shodex RI-101, Showa Denko, Japan). The pure CAs (0.001−0.2%, w/v) were dissolved in distilled water and filtered through a 0.45 μm syringe filter. Then, the CA mixture was separated by Shodex OHpak SB-804 HQ and OHpak SB-802.5 HQ columns (8.0 mm inner diameter × 300 mm each, Showa Denko, Japan) in tandem. The columns were maintained at 50 °C, and deionized water (18.2 MΩ cm) was used as an eluent at a flow rate of 0.8 mL/min.[11] On the basis of the peak area of each CA concentration, the standard curve was prepared. The production yield of CAs from various 4αGTase reaction conditions was quantified using the CA standard curve under the same HPSEC operating method.[11]

One-Pot Synthesis of CAs by Dual Enzyme Treatment. The biocatalytic production of CAs was carried out with 0.3 M sucrose in 10 mL of 50 mM sodium phosphate buffer (pH 7.0) up to 12 h. In this one-pot synthesis of CAs, the enzymatic reaction was conducted by simultaneous treatment of both NpAS (2.5−20 units/mL) and 4αGTase (0.1 unit/mL) at 35 and 40 °C. During the reaction, an aliquot of the reaction mixture (1.0 mL) was taken at designated time intervals (0, 1, 3, 5, 7, 9, and 12 h) and inactivated in boiling water for 10 min. After cooling, it was centrifuged at 9000*g* for 10 min to remove the denatured enzyme protein and insoluble linear α-(1,4)-glucan products. Then, 0.1 mL of the supernatant was mixed with 0.4 mL of 50 mM sodium acetate buffer (pH 5.5) and treated with glucoamylase (0.4 unit/mL) for hydrolyzing the residual linear α-(1,4)-glucans into glucose. Once the final reaction mixture was precipitated with the addition of 10-fold ethanol, the highly purified CAs were obtained.[7] The conversion yield of CAs based on the initial amount of sucrose was calculated from the peak area of HPSEC analysis using the standard curve generated in this study. In the sequential enzyme treatment method, the only difference in the reaction condition from the simultaneous treatment above was the time point of 4αGTase treatment after the NpAS reaction was initiated for chain elongation from sucrose. The NpAS reaction was conducted first for the prescribed time periods (0, 1, 3, 5, 7, 9, and 12 h), and then 4αGTase (0.1 unit/mL) was added to the pre-reacted mixture for another 2 h reaction.

Two-Step Enzymatic Synthesis of CAs. In the first step of CA production, linear α-(1,4)-glucans were synthesized *in vitro* by NpAS treatment. To obtain various sizes of linear α-(1,4)-glucans, a wide range of the sucrose concentration (0.05−1.0 M) was used in 10 mL of 50 mM Tris-HCl buffer (pH 7.0) for the NpAS reaction (12 units/mL) at 35 °C for 24 h. The reaction products as precipitate form were separated by filtration (Whatman filter paper no. 2; 8 μm pore size) and finally recovered by freeze-drying.[15] Production yields of linear α-(1,4)-glucans were calculated on the basis of the initial amount of sucrose as a substrate. Molecular size distributions of the linear α-(1,4)-glucan products were analyzed by HPSEC. Shodex OHpak SB-804 and SB-802.5 analytical columns were applied to a HPSEC system (Summit). The flow rate was 1.0 mL/min, and deionized water (18.2 MΩ cm) was used as a mobile phase. For the sample preparation in the HPSEC analysis, the linear α-(1,4)-glucan products were dissolved by the same dissolving method of potato amylose aforementioned. Once the ethanol-precipitated α-glucans were solubilized in 1 mL of deionized water by heating for 1 h, the solution was immediately filtered through a 0.45 μm membrane and injected into the HPSEC system. Shodex pullulan P-82, maltopentaose (G5), and maltoheptaose (G7) were employed as standards.[11]

As the second step of enzymatic CA synthesis, the CAs were produced from linear α-(1,4)-glucans by 4αGTase, as described above. In this experiment, 10 mg of the dissolved linear α-(1,4)-glucan products in 1.0 mL of 50 mM sodium phosphate buffer (pH 7.0) was treated with 4αGTase (0.1 unit/mL) at 45 °C for 2 h.[11] Quantification of the purified CAs was performed by HPSEC analysis, as described above, and the production yields of CAs were calculated accordingly.

Data Fitting and Analysis. A modeling approach that fitted experimental data to a conceived mathematical equation was used to evaluate the relationships of the sucrose concentration ([Suc]), glucan chain length, and CA yield. Experimental observation that showed an asymptotic decay in DP with respect to [Suc] was fitted using an exponential function (eq 1)

$$\mathrm{DP} = Ae^{-\alpha[\mathrm{Suc}]} \tag{1}$$

where A and α are coefficients to be estimated using curve fitting.

To elucidate the relationship between the CA yield and DP, we used eq 2 that produced a saturation curve to fit the experimental data for the CA yield against DP, which showed saturation of the CA yield as increasing DP

$$\text{yield (\%)} = (V \cdot \mathrm{DP})/(K + \mathrm{DP}) \tag{2}$$

where V is the maximum yield (%) and K is the DP value at the half-maximum yield. Values for V and K were estimated using curve fitting.

In the curve-fitting procedure, the optimized parameter values that minimized the error caused by the discrepancy between actual data and model prediction were numerically estimated.

Subsequent analysis was then performed to find the optimum relationship among [Suc], DP, and CA yield. For this analysis, we divided the data into two sections: linear (increase or decay) and saturate regions. Two-component regression analysis was applied to separately fit the data in two sections by regression lines for searching the optimal CA yield by [Suc] and DP. MATLAB (version 7.9.0.529, The Mathworks, Inc., Natick, MA) was used for all of the data analyses, including the curve-fitting and optimizing parameter values.

RESULTS AND DISCUSSION

Enzymatic Production of Highly Pure CAs from Potato Amylose. To obtain a standard mixture of CAs, commercial potato amylose was used as a substrate for the 4αGTase reaction. The purity of CA products was evaluated by MALDI−TOF MS analysis after glucoamylase treatment, and it was confirmed that no linear glucans remained in the CA products (Figure 1A). On the MALDI−TOF mass spectrum, the relative peak intensity of cyclic DP 26 (M_w of $[\mathrm{CA}_{26} + \mathrm{Na}]^+ = 4237$) was greatest and the minimum detectable peak of cyclic DP was 21. In the 4αGTase reaction process, the production yield of CAs was investigated in the temperature range of 35−45 °C up to 3 h (Figure 2). From all of the temperatures tested, the maximum CA yields were identically obtained at 2 h of reaction with 0.1 unit/mL of 4αGTase and then the yield decreased thereafter. Meanwhile, the production rate and maximum yield of CAs increased as the reaction temperature increased.

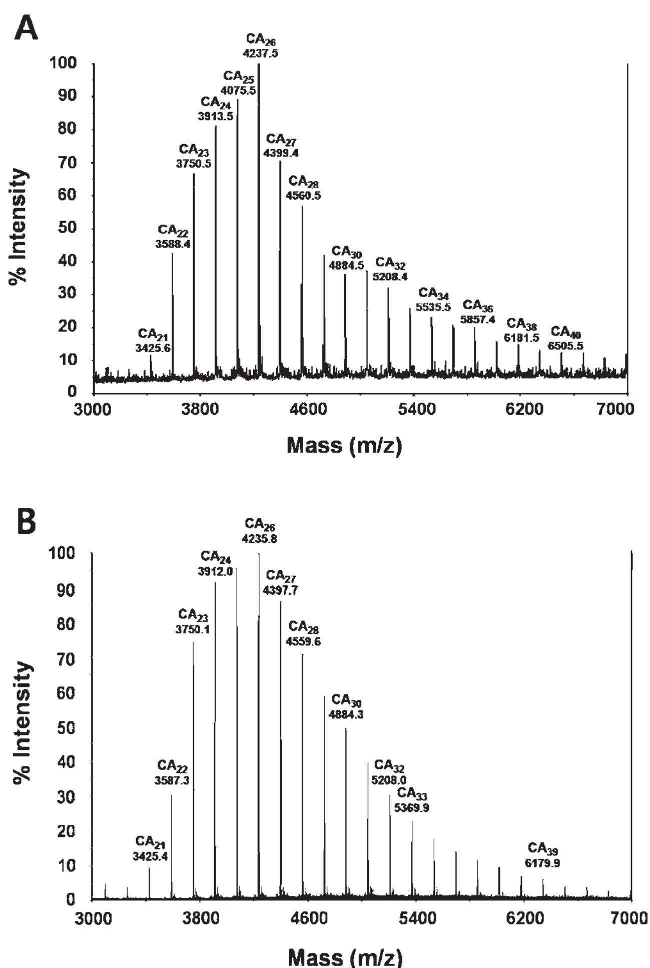


Figure 1. MALDI−TOF MS analysis of CAs produced with (A) potato amylose at optimal reaction conditions of 4αGTase and (B) sucrose at optimal one-pot synthesis reaction conditions. The subscript "n" on CA_n indicates the degree of polymerization of CAs. The number above each peak indicates the molecular mass (in daltons) of the molecule plus 23 Da (sodium ion).

A HPSEC calibration curve was generated using the highly purified CA standard mixture (Figure 3), which was used for the quantification of CA products from various enzyme reaction conditions in this study. On the HPSE chromatogram, the apex of each CA peak was constantly eluted at 20 min, which was equivalent to DP 19. However, the size of these cyclic glucans cannot be directly compared to the linear glucan standards in the HPSEC system. It is well-known that linear molecules elute out earlier than the spherical forms in the HPSEC system when the

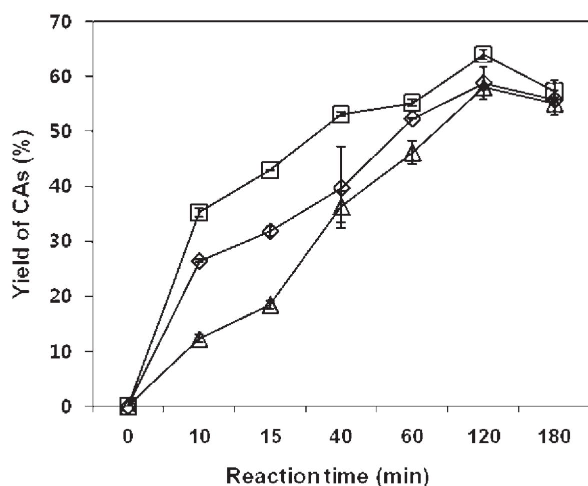


Figure 2. Effect of the reaction temperature on the CA yield from potato amylose by 4αGTase. A total of 0.1 unit of 4αGTase activity was used per 1 mL of the reaction volume. The CA yield was calculated on the basis of the initial amount of potato amylose as a substrate. The symbols in the graph indicate the reaction temperatures of (△) 35 °C, (◇) 40 °C, and (□) 45 °C.

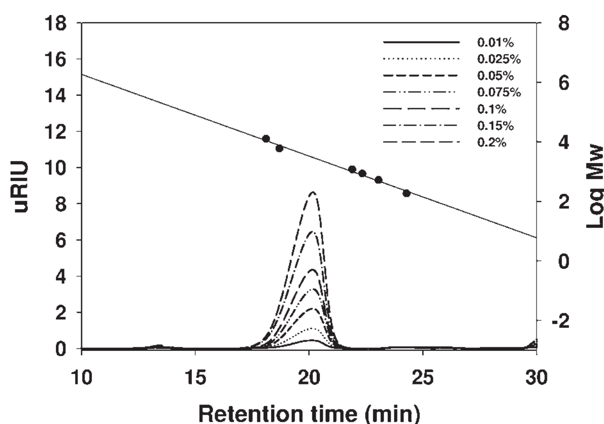
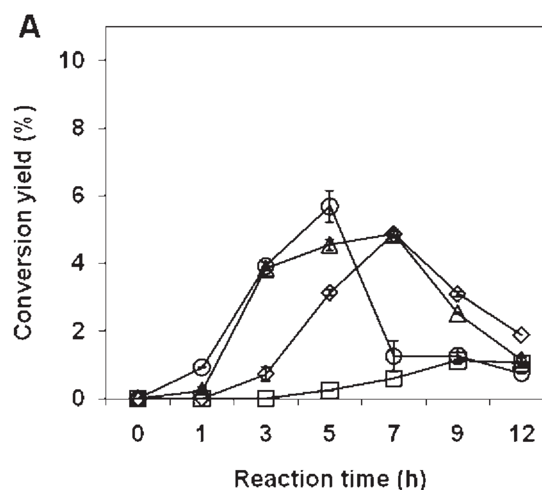


Figure 3. HPSEC analysis of the CA standard mixture (0.01−0.2%) produced from potato amylose by 4αGTase treatment. The standards used for the calibration curve are P10, P5, G7, G5, G3, and G1.



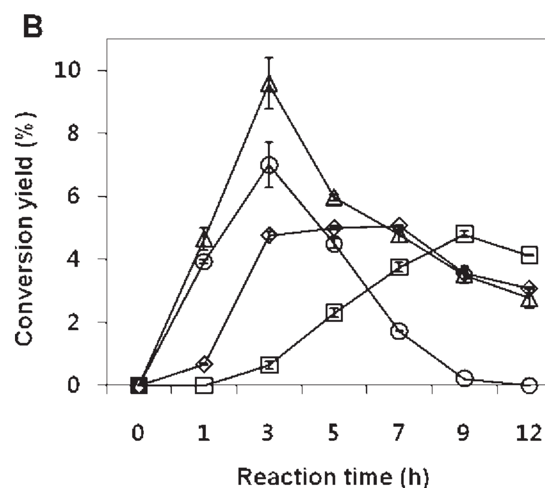


Figure 4. Bioconversion yields of CAs with 0.3 M sucrose as a substrate by the simultaneous reaction mode at (A) 35 °C and (B) 40 °C. The symbols in the graph indicate the NpAS activities of (□) 2.5 units/mL, (◇) 5.0 units/mL, (△) 10 units/mL, and (○) 20 units/mL.

molecular weight of both compounds is the same.[22] It was confirmed by analyzing β-cyclodextrin (cyclic DP7) and maltoheptaose (G7) (data not shown). Thus, the discrepancy in the major cyclic glucan size occurred between the results of MALDI−TOF and HPSEC analyses. The intensity of the refractive index (RI) response of the CA standard mixture was linearly correlated with its amount in a perfect manner up to 0.2% (w/v; R^2 = 0.999).

One-Pot Synthesis of CAs by Simultaneous Dual Enzyme Treatment. In this experiment, one-pot synthesis of CAs was conducted by simultaneous treatments of both NpAS and 4αGTase. Theoretically, NpAS produces linear α-(1,4)-glucans from sucrose as the sole substrate, from which the CAs are synthesized by the 4αGTase reaction. This reaction was carried out with 0.3 M sucrose in a 50 mM phosphate buffer (pH 7.0) up to 12 h. The resulting reaction mixture was treated with glucoamylase for hydrolyzing the residual linear α-(1,4)-glucans as a purification step. Once the final reaction mixture was precipitated with adding 10 volumes of ethanol, the highly purified CAs were obtained. The conversion yield of sucrose to CA products was determined by HPSEC. The initial rate of CA formation increased in general as the activity ratio of NpAS/4αGTase increased (panels A and B of Figure 4). However, too much NpAS activity over 4αGTase eventually dropped the conversion yield to CAs. At 40 °C, the initial and maximal CA yields with 20 units/mL NpAS and 0.1 unit/mL 4αGTase were less than those with 10 units/mL NpAS and 0.1 unit/mL 4αGTase. Therefore, the activity ratio of NpAS and 4αGTase would be a very important factor to optimize CA production with this reaction system. It has been well-known that linear α-(1,4)-glucans easily associate with each other and eventually precipitate out from aqueous solution.[17] Thus, the glucan elongation rate by NpAS should be controlled to keep amylose-like products in the solution. The greater production yield of CAs was achieved at 40 °C than at 35 °C, and the maximum yield of CAs (9.6%) was obtained with 10 units/mL NpAS and 0.1 unit/mL 4αGTase at 40 °C for 3 h of reaction (Figure 4B). This result is partially explained by the increase in the solubility of linear glucans because of the increase in the reaction temperature.

One-Pot Synthesis of CAs by Sequential Dual Enzyme Treatment. In this biocatalytic process, NpAS was added to sucrose substrate solution first, and then after designated reaction times, 0.1

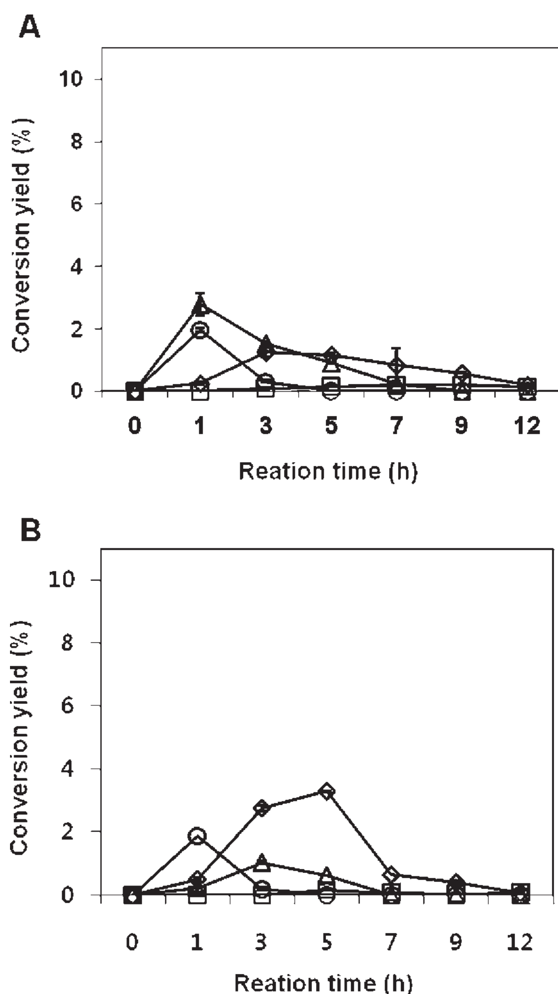


Figure 5. Bioconversion yields of CAs with 0.3 M sucrose as a substrate by the sequential reaction mode at (A) 35 °C and (B) 40 °C. The values on the *x* axis indicate the reaction time periods of NpAS before adding 4αGTase for another 2 h of reaction, and the symbols in the graph indicate the NpAS activities of (□) 2.5 units/mL, (◇) 5.0 units/mL, (△) 10 units/mL, and (○) 20 units/mL.

unit/mL 4αGTase was applied for another 2 h. When 4αGTase was added after 1 h of NpAS treatment at 35 °C, the maximum yields of CAs reached 2.8 and 2.0% with 10 and 20 units/mL of NpAS, respectively, in the time course study (Figure 5A). With 5 units/mL of NpAS at the same reaction conditions, the greatest yield (1.3%) was obtained after 3 h of reaction and then the yield gradually decreased along with NpAS treatment time. At both 35 and 40 °C, no discernible amount of CAs was produced with 2.5 units/mL NpAS. When the reaction temperature was raised to 40 °C, the greatest yield of CAs (3.3%) was observed from 5 h of reaction with 5 units/mL NpAS. More than this level of enzyme dosage resulted in an adverse effect on the CA yields. As noticed from the individual CA yield patterns on the graphs of both simultaneous and sequential reaction modes, the CA yields decreased from all of the treatment groups with various rates after the CA production reached a top from both of the reaction temperatures. It has been reported that 4αGTase may hydrolyze its own CA products,[23,24] resulting in a lower CA yield. On the other hand, the elongation rate of NpAS was dominant with either a longer reaction time or a larger amount, which resulted in precipitation of linear glucan products by chain−chain

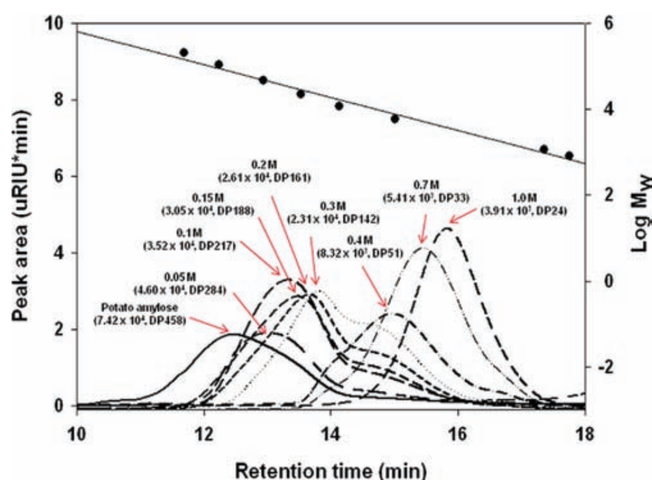


Figure 6. Molecular-weight distributions of linear α-(1,4)-glucans synthesized with NpAS from various sucrose concentrations by HPSEC. The standards used for the calibration curve are P200, P100, P50, P20, P10, P5, G7, and G5.

association, and then these precipitates cannot be used as substrate for the 4αGTase reaction any more. Optimum activity temperatures of NpAS and 4αGTase were 35 and 45 °C, respectively, as previously reported.[11,25] In comparison to the CA production rate at 35 °C, the relative NpAS activity decreased and the 4αGTase activity increased at 40 °C. Thus, the precipitate formation rate by NpAS decreased, which resulted in greater CA production. A higher CA production yield was obtained at 40 °C than at 35 °C when the same activity of NpAS was applied. When the purified CAs produced from the optimum reaction condition were analyzed by MALDI−TOF MS, the relative peak intensity profile of cyclic DPs was almost identical to that of CAs synthesized with potato amylose (panels A and B of Figure 1) and no presence of linear glucans was also confirmed.

Two-Step Synthesis of CAs by Enzyme Treatment. Linear α-(1,4)-glucans were produced first from sucrose substrate by NpAS treatment in this two-step process of CA production. As a result, a series of linear glucan sizes were obtained from various sucrose concentrations, and the molecular size distributions of these linear α-(1,4)-glucan products were analyzed using HPSEC (Figure 6). The NpAS reaction products were retrieved from 0.1 to 1.0 M sucrose as a white precipitate form, which was used as substrates for CA synthesis after DMSO-assisted solubilization. The glucan product from 0.05 M sucrose was unable to be retrieved as a precipitate form. Thus, the ethanol-precipitated product was recovered and used as a substrate for CA production. Even though the molecular size of this product was the greatest ($M_w = 4.60 \times 10^4$), these linear α-(1,4)-glucans were unlikely to precipitate by chain−chain association because of the insufficient critical mass in the reaction solution. The production yields of linear glucans from various sucrose concentrations are presented in Table 1. Commercial potato amylose was also used for the 4αGTase reaction substrate. The peak M_w of this natural biomacromolecule was 7.42×10^4, which is equivalent to DP458. On the basis of the standard calibration curve prepared in this study, the molecular size of each linear α-(1,4)-glucan product was represented by the DP at the apex of the peak in the chromatogram. As a result, it was found that [Suc] was inversely related to the DP of linear glucan products (Figure 7), as expected from the previous report.[15] At the greatest [Suc] = 1.0 M, the DP of the glucan product was 24, while DP 284 was

Table 1. Production Yields of Linear α-(1,4)-Glucans and CAs Synthesized from Various Sucrose Concentrations

sucrose concentration (M)	yield of linear α-(1,4)-glucans (mg/10 mL)	yield of linear α-(1,4)-glucans (%)[a]	yield of CAs (%)[a]
0.05	53.4 ± 1.0	31.2	17.3
0.1	84.8 ± 2.2	24.7	14.9
0.15	119.5 ± 9.3	23.2	11.7
0.2	186.2 ± 8.6	27.1	13.8
0.3	279.5 ± 14.9	27.2	13.5
0.4	315.8 ± 7.9	23.1	9.9
0.7	405.5 ± 17.4	16.9	4.2
1.0	744.7 ± 7.1	21.8	4.9

[a] The yields were calculated on the basis of the initial amount of sucrose as a substrate.

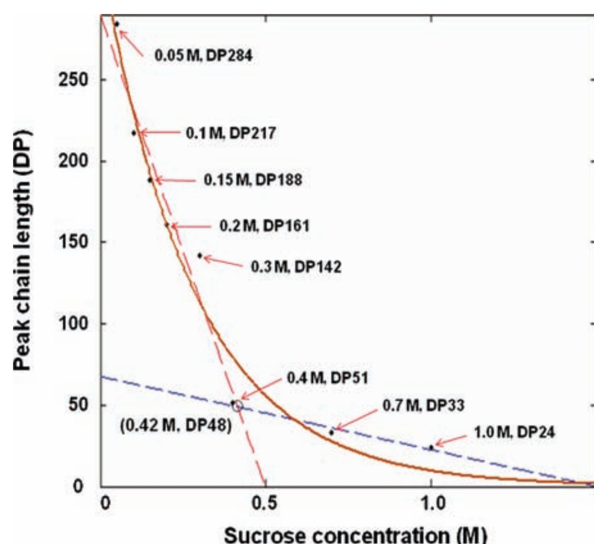


Figure 7. Fitted curve that shows the relationship between the peak chain length and sucrose concentration. The solid line indicates the fitted curve by the nonlinear eq 3, while the broken lines indicate two-component regression traces. The intersecting point is shown as an open circle.

obtained from [Suc] = 0.05 M. The [Suc] strongly affected the DP of insoluble glucan products by NpAS treatment, which means that the critical glucan size precipitating out is totally dependent upon [Suc].

Optimal parameter values were estimated by fitting the exponential function to experimental data that observed the relationship of [Suc] and DP (eq 3).

$$DP = 327.4e^{-3.537[Suc]} \quad (R^2 = 0.9653) \qquad (3)$$

In eq 3, the coefficient in the power term represents the decay rate of DP with respect to [Suc], indicating that the increase in [Suc] asymptotically reduces DP. Theoretically, the upper limit of the DP of synthesized glucan would be around 327 when [Suc] infinitely approaches 0 under this reaction condition. Two-component regression lines and the intersection located at (0.42 M, DP 48) are also shown in Figure 7. As the [Suc] decreased from 1.0 M, the DP of glucan products increased gradually. Once [Suc]

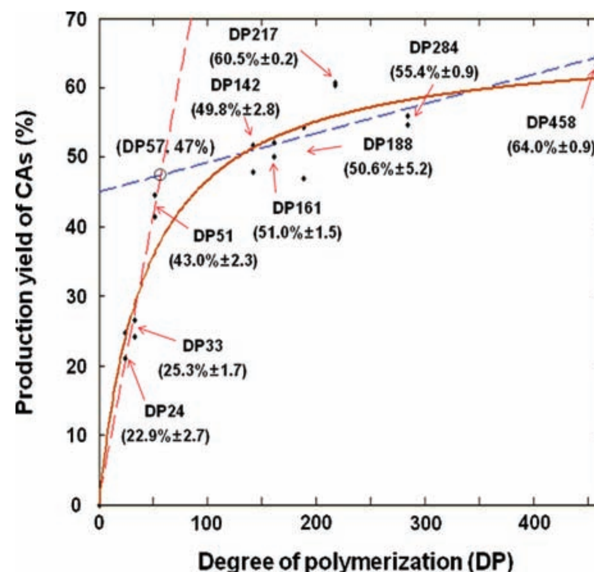


Figure 8. Production yields of CAs from various sizes of linear α-(1,4)-glucans. The relationship between the DP and CA yield was fitted by eq 4. Notations are the same as shown in Figure 7.

reached around 0.42 M and below, the DP started to increase drastically. Longer glucan chains are more stable without precipitation in lower [Suc], which can still be used as a substrate for the 4αGTase reaction. This result can be used to produce various sizes of amylose-like glucan products under this enzymatic process.

Using these size-defined glucan products, the substrate preference of *Synechocystis* 4αGTase was evaluated in terms of CA productivity, which resulted in the longer glucan chains being more preferable to CA production with the 4αGTase treatment (Figure 8). Meanwhile, the relationship of the CA production yield against the DP of glucan was fitted using a nonlinear function (eq 2) and resulted in the following eq 4:

$$\text{yield of CAs (\%)} = (67.15DP)/(43.69 + DP) \qquad (4)$$

The maximum CA yield estimated by eq 4 was 67.2%. Two-component regression was also applied to find the intersection, which turned out to be (DP 57, 47%). Thus, this relationship showed a transition point at DP 57. Up to DP 57 of the glucan substrate, the CA yields increased drastically along with the size of glucans, while above DP 57, the increase rate of the CA production yield substantially decreased. That is, a change in the CA yield per a unit change in DP was drastically reduced beyond DP 57. The CA yield reached about 70% of the maximal yield, i.e., 47.4% of 67.2%, with only increasing DP by 57. In contrast, the remaining 30% of the maximum CA yield can be accomplished when DP increases larger than 458. It suggests that the size of linear glucan may be one of the important factors to determine the production yield of CAs.[26] Final production yields of CAs from sucrose substrate in the range of 0.05−1.0 M were calculated and presented in Table 1 as well.

Efficiency Comparison of Enzymatic Process Methods for CA Production. Using commercial amylose as a substrate for CA synthesis by 4αGTase, the production yield was 64% at the optimum reaction conditions in this study (Table 2). However, this huge biomacromolecule is not readily dissolved, and an additional solubilization process should be involved in the CA production. A limitation on the increase in the substrate

Table 2. Optimum Reaction Conditions for the Maximum Production Yield of CAs

substrate	reaction mode	reaction temperature (°C)	4αGTase (unit/mL)	NpAS (units/mL)	reaction time (h)	maximum yield of CAs (%)[a]
1% potato amylose	single	45	0.1	0	2	64.0 ± 0.9
0.3 M sucrose	dual, sequential	40	0.1	5	5	3.4 ± 0.0
0.3 M sucrose	dual, simultaneous	40	0.1	10	3	9.6 ± 0.8

[a] The maximum yields were calculated on the basis of the initial amount of either potato amylose or sucrose as a substrate.

concentration is a critical drawback in this case; thus, only 6.4 g of CAs/L of reaction mixture can be produced from 1% solution of amylose. In comparison to it, the one-pot synthesis method adopted in this study seemed to show a lower maximal CA yield (9.6%) from 0.3 M sucrose (Table 2); however, sucrose is highly soluble, and a concomitant dual enzyme reaction led to the conversion of simple sugar directly to CAs via transient linear glucans. With 102.6 g of sucrose in 1 L of solution, 10.1 g of CAs can be produced. Even the remaining sucrose after the reaction can be recycled for the next production process of CAs. Therefore, this newly developed bioprocess showed great potential to produce CAs efficiently on an industrial scale.

For the first time, we reported an efficient bioconversion process of CA production by one-pot enzymatic synthesis in this study. This process did not require a substrate-dissolving step; thus, it simplified the process for CA synthesis. Starting with sucrose as an initial substrate, in the two-step enzyme treatment for CA production, the yield of α-(1,4)-glucan was in the range of 17−31% and, in turn, the final production yield of CAs was calculated to be 4−17% (Table 1). Considering the difficulty in the solubilization of linear α-(1,4)-glucans, one-pot synthesis of CAs by this dual enzyme treatment would be an innovative bioprocess. In the NpAS reaction, it was clearly shown that the chain length of α-(1,4)-glucan products decreased with an increasing [Suc] in the reaction mixture. This obvious opposite effect of [Suc] on the chain length of α-(1,4)-glucan products can be used to produce an expected size of amylose-like glucan products by controlling [Suc]. The production yield of CAs in the 4αGTase reaction was strongly affected by the chain length of α-(1,4)-glucan substrates. Therefore, the substrate preference of 4αGTase should be considered to optimize the CA production from this enzymatic bioprocess system. Other 4αGTases from various bacterial species can be used to improve the productivity of CAs as well.

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