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Expression, purification, and characterization of a novel amylosucrase from *Neisseria subflava*

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ABSTRACT

Amylosucrase (ASase) is a glucosyltransferase, which catalyzes the *de novo* synthesis of amylose-like polymers from sucrose. In the present study, ASase from *Neisseria subflava* (*Ns*AS) was cloned, sequenced, and expressed in *Escherichia coli*. The production of *Ns*AS was achieved by inducting gene expression with 0.2 mM isopropyl-β-D-thiogalactopyranoside. The molecular mass of the Ni-NTA column purified *Ns*AS analyzed by SDS-PAGE was determined to be 72 kDa. *Ns*AS exhibited maximal activity at 45 °C and pH 8.0, and showed strong thermal stability at 40 °C with a half-life of 385 h. The reaction pattern of *Ns*AS at [sucrose] range of 0.1–1.0 M showed that at 0.7 M of [sucrose], the production yield of insoluble linear α-(1,4)-glucans reached 24% maximum, and any further increase in [sucrose] resulted in a slight decrease in yield. Meanwhile, the production yield of turanose significantly increased from 16 to 29% by increasing [sucrose] from 0.1 to 1.0 M. The synthesized glucan had degrees of polymerization (DP); for 0.1, 0.4, 0.7, and 1.0 M sucrose, the DP values were 77, 49, 39, and 31 respectively. These results suggested that *Ns*AS would be a promising candidate for food industrial production of linear α-(1,4)-glucans and turanose as a next generation sweetener.

1. Introduction

Amylosucrase (ASase) (E.C. 2.4.1.4) is an α-glucosyltransferase from glycoside-hydrolase (GH) family 13 which catalyzes the *de novo* biosynthesis of amylose-like polymers (α-1,4-glucan) using sucrose as a substrate and unique energy source, releasing fructose [1–4]. ASase catalyzes the α-D-glucosyl residue transfer from sucrose to the 4-position of the non-reducing end of an α-glucan, producing an insoluble α-(1,4)-glucan accompanied by the release of D-fructose from sucrose. ASase also has sucrose hydrolytic activity to release D-glucose and D-fructose molecules and may catalyze the transglycosylation reaction from sucrose to many acceptor molecules. In addition, ASase uniquely produces a small amount of sucrose isomers, including turanose and trehalulose when the D-glucosyl moiety of sucrose is transferred onto the released D-glucose and D-fructose, respectively [1–7]. In contrast with other amylopolysaccharide synthases, ASase does not require any expensive nucleotide-activated sugars (such as ADP- and UDP-glucose) as a glucosyl donor for the transglycosylation reaction [4,5]. Indeed, the energy for the synthesis of a new α-1,4-glycosidic bond is obtained from the breaking down of the α1-β2 linkage of sucrose [4,6]. Therefore, ASase can be considered as a smart enzyme suitable for industrial biosynthesis and modification of amylopolysaccharides [4,5,7].

ASase was initially isolated from Gram-negative, non-pathogenic bacteria, *Neisseria perflava,* as an extracellular enzyme [8]. *N. polysaccharea* also has an extracellular ASase [9]. So far, only five recombinant ASase, *Np*AS from *Neisseria polysaccharea* [2], *Dr*AS from *Deinococcus radiodurans* [10], *Dg*AS from *Deinococcus geothermalis* [11], *Am*AS from *Alteromonas macleodii* [12], and *Arthrobacter chlorophenolicus* [13], have been characterized. Among ASases, *Np*AS has been the most widely studied ASase [14,15]. *Np*AS was the only one with a known crystallographic structure [2] until the structure of *Dg*AS [11] and *Dr*AS [16] were determined. ASase is composed of the three common domains: a catalytic $(\beta/\alpha)_8$-barrel folded domain called A-domain, the B-domain which is the loop 3 extension of the catalytic barrel, and the C-terminal domain. Moreover, if an acceptor was added to the reaction, the ASase had a preference to pair the D-glucose residue onto the non-reducing end of acceptors instead of yield linear α-1,4 glucans. Therefore, the elongated polymers were highly digestion-resistant through glucan-chain association. As a result of enzymatic modification, retrograded, starch (RS3) was formed at the end [17,18,19]. A series of corn, rice, and barley starches acted

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as acceptors, and thus they were modified by *Np*AS treatment [17,19]. The RS contents in *Np*AS-modified starches significantly increased compared to untreated native starches. Moreover, *Np*AS can synthesize sucrose isomer (turanose, trehalose) [15,20]. This reaction was preferably catalyzed by *Np*AS at a relatively high concentration of substrate, while the biosynthesis of glucan was inhibited. Therefore, the purpose of the present work was to clone the gene corresponding to the ASase from *N. subflava* NJ9703 (*Ns*AS) and express this in *Escherichia coli*. Further, the purification and characterization of the recombinant *Ns*AS is a prerequisite for understanding their role in the production of α-1,4-glucan and turanose.

2. Materials and methods

2.1. Bacterial strains and culture conditions

N. subflava NJ9703 (ATCC 49275) was used as a source of the *AS* gene. *E. coli* DH5α was used as a host for typical DNA manipulation, while *E. coli* BL21-CodonPlus RIL [F^-, ompT, hsdSB (rB^-, mB^-), dcm, gal, λ (DE3)] from Agilent Technologies Co. (Carpinteria, CA) was used as a host for expression.

E. coli strains were routinely cultivated at 37 °C in LB medium, supplemented with the antibiotics, Ampicillin. For the ASase production, the transcription was induced by addition of IPTG (isopropyl-β-D-thiogalactopyranoside) at 0.2 mM when Abs_{600} of 0.6–0.8 was reached. Cells were harvested after additional incubation at 30 °C for 18 h.

2.2. Database search and sequence analysis

A database of ASase was obtained from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). The genomic DNA of *N. subflava* NJ9703 (NCBI accession number: ACEO02000010.1) revealed that a putative *ams* gene encoding an enzyme AS was found in the genome (gene locus_tag: NEISUBOT_05048, protein ID: EFC51554.1). Multiple sequence alignment was performed using NCBI's COBALT-constraint-based multiple alignment online tool to compare the homology of the amino acid sequences based on *Np*AS. The biosafety level of AS producing bacteria was also checked using the American Type Culture Collection (ATCC, http://www.atcc.org) and the Bacterial Diversity Meta-database (BacDive, http://bacdive.dsmz.de).

2.3. Gene isolation and vector construction

The genomic DNA of *N. subflava* NJ9703 revealed that a putative *ams* gene encoding an enzyme ASase was found in the genome (gene locus_tag: NEISUBOT_05048, protein ID: EFC51554.1). Further, gene synthesis was performed by the AllInOneCycler™ 384 well PCR system (Bioneer Co., Daejeon, Korea). The gene corresponding to *Ns*AS was amplified with *Pfu* DNA polymerase (Fermentas, St. Leon-Rot, Germany), using the forward primer 5′ GGAGCTAGCATTATGTTGACCCAGACTC 3′ and the reverse primer 5′ TAAACTCGAGAGCTTATGCAATTTCAAGCC 3′ with two restriction sites, NdeI and Xho1 respectively. The PCR amplification was performed on a PCR Thermal Cycler (Bio-Rad). The PCR conditions for each PCR reaction was as follows: 50 ng template DNA, 1.0 mM of each primer, 0.25 mM of each dNTP, 5 × DNA polymerase buffer, and 1 U DNA polymerase. The PCR steps were composed of an initial denaturation step for 2 min at 95 °C, 25 cycles of 30 s at 95 °C, 30 s at 50 °C, 1 min at 72 °C for amplification, and the last elongation step at 72 °C for 7 min.

The resulting *Ns*AS gene product was cloned into the pBT7-N-His expression vector (Bioneer Co.). DNA sequencing analysis was carried out by using ABI3730XL sequencer (Applied Biosystems) using T7 promoter/terminator primers. The sequenced *Ns*AS was compared with the original *Ns*AS sequence in the NCBI database. The final construct was named pBT7-*Ns*AS.

2.4. Gene expression

The constructed recombinant vector was transformed into *E. coli* BL21 (DE3) for efficient heterologous protein expression. The IPTG induction was started by growing the *E. coli* BL21 (DE3) cells harboring pBT7-N-His-*Ns*AS in 1.0 L of LB medium, supplemented with 50 μg/mL of ampicillin, at 37 °C with agitation until the optical density reached 0.6–0.8 at 600 nm. Then IPTG was added to a final concentration of 0.2 mM followed by an 18 h growth period at 18 °C to induce the expression of the chimeric gene. The cells were harvested by centrifugation at 5,000*g* and 4 °C for 10 min. The cells were then re-suspended in a 50 mM Tris-HCl buffer (pH 8.0). The bacterial pellet was re-suspended in a binding buffer and disrupted by sonication (VibraTM Cell VC 750; Sonics & Materials, Inc, Newtown CT) in an ice bath. The supernatant acquired by centrifugation (10,000*g* at 4 °C for 20 min) was used as a starting material to obtain recombinant proteins. The supernatants containing *Ns*AS were filtered through a 0.45-μm syringe filter and the supernatant was harvested for subsequent purification.

2.5. Purification and electrophoresis

The process of purification of $(His)_6$-tagged *Ns*AS was conducted in a nickel- nitrilotriacetic acid (Ni-NTA) affinity chromatography column (Qiagen, Hilden, Germany). The buffer of eluent was changed to a 50 mM Tris-HCl buffer (pH 8.0) and the enzyme solution was concentrated through centrifugation (2,500*g*) at 4 °C for 20 min by using Amicon Ultra-15 centrifugal filters of 30 K (Merck Millipore, Carrigtwohill, Ireland). The activity of *Ns*AS was measured by the DNS method using fructose as a standard. Protein concentration was determined according to the Bradford method [21] using Bovine serum albumin as a standard.

The molecular mass of ASase was measured by electrophoresis in 12% polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS-PAGE) as described by Laemmli [22]. The molecular weight of the protein was determined by comparing the migration of the standard molecular weight protein in the same gel. Protein bands were visualized with Coomassie brilliant blue R-250 (Bio-Rad) staining.

2.6. Characterization of recombinant NsAS

2.6.1. Determination of enzyme activity

*Ns*AS were assayed by the method proposed by Wang et al. [15]. *Ns*AS activity was measured by determining the amount of reducing sugar that was produced from sucrose hydrolysis. The enzyme assay was carried out in a 50 mM Tris-HCl buffer (pH 8.0) containing 0.1 M sucrose at 40 °C for 30 min. After incubation, the reaction was terminated by adding 0.5 mL of DNS reagent and boiling the mixture in a water bath for 5 min. The absorbance of the solution was measured as 575 nm. One unit of *Ns*AS was defined as the amount of enzyme that releases 1.0 μ mol of fructose per min under the assay conditions.

2.6.2. Effect of pH and temperature on enzyme activity

The optimal pH for enzyme activity was determined to lie in the range of 4–10. Four buffer systems were used for analyzing the effect of pH on enzyme activity: a sodium acetate buffer (50 mM, pH 4.0–6.0); a sodium phosphate buffer (50 mM, pH 6.0–7.5); a Tris-HCl buffer (50 mM, pH 7.0–9.0); and glycine-NaOH buffer (50 mM, pH 8.0–10.0). The optimal pH for enzyme activity was determined by the DNS method [15]. The effects of temperature on enzyme