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An unusual chimeric amylosucrase generated by domain-swapping mutagenesis

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ABSTRACT

Amylosucrase (ASase; EC 2.4.1.4) synthesizes α-1,4-glucans using sucrose as a sole substrate. The aim of this study was to compare the enzymatic properties of four recombinant *ASase* genes to determine the underlying mechanisms thereof. Following cloning and expression in *Escherichia coli*, we determined that the ASase enzyme from *Deinococcus geothermalis* (DGAS) had the highest thermostability whereas ASase from *Neisseria polysaccharea* (NPAS) showed the greatest polymerization activity. Chimeric ASases were constructed using *dgas* and *npas* genes by overlap extension polymerase chain reaction. Two of the six chimeric ASases generated, NPAS-B′ and DGAS-B, showed ASase activity using sucrose as the sole substrate. However, DGAS-B was not able to produce longer α-1,4-glucans; the highest degree of polymerization was <12. In the kinetic study, not only the substrate binding affinity but also the production rate of DGAS-B was greater than those of DGAS. Molecular dynamic computational simulation suggested that DGAS-B could not synthesize longer glucan chains because of the change in flexibilities of loops 4, 7, and 8 as compared to those of DGAS.

1. Introduction

Amylosucrase (ASase; EC 2.4.1.4) synthesizes α-1,4-glucans from sucrose as the sole substrate [1,2]. ASase also catalyzes sucrose hydrolysis, releasing glucose and fructose; the synthesis of α-1,4-oligosaccharide uses the released glucose as an acceptor, and the minor production of sucrose isomers such as trehalulose and turanose uses the released fructose as an acceptor [3]. As DNA sequencing technology has become more advanced, various ASase genes have been cloned, including those from *Neisseria polysaccharea* (NPAS), *Deinococcus radiodurans* (DRAS), *Deinococcus geothermalis* (DGAS), *Alteromonas macleodii* (AMAS), and *Arthrobacter chlorophenolicus* (ACAS) [4–8]. In addition, it has recently been found that ASase can utilize many molecules other than α-1,4-glucan as an acceptor, including glycoside compounds such as galactose, xylose, salicin, and arbutin, as well as aglycone compounds such as catechin and hydroquinone [6,9–12].

ASase belongs to glycoside hydrolase (GH) family 13 based on its amino acid sequence [13]. Generally, the three-dimensional structure of GH13 family enzymes consists of domain A, containing a catalytic $(\beta/\alpha)_8$-barrel, domain B, which is inserted in the loop between the β3-strand and α3-helix of the barrel, and domain C, which includes an antiparallel β-sheet at the C-terminus [14]. Among the various ASases, the three-dimensional structures of NPAS, DGAS, and DRAS have been solved [3,15,16]. Unlike other GH13 family enzymes, ASase consists of five domains named N, A, B, B′, and C. The N domain, consisting of about 90 residues, which form five helices at the N-terminus, and the B′ domain, which is inserted between the β7-strand and α7-helix of the $(\beta/\alpha)_8$ domain are only found in ASase, whereas the three other domains are conserved among GH13 family enzymes. Notably, the topology of the A, B, and B′ domains of ASase form a unique active pocket, giving rise to its polymerization activity [3,17]. When the inactivating mutant NPAS-E328Q is crystallized with sucrose or maltoheptaose, two sucrose binding sites (SB1 and SB2) and three oligosaccharide binding sites (OB1-3) are observed [17]. SB1 and OB1 span the −1 and +1 subsites in the active site, and SB2, OB2, and OB3 are positioned on the surface of the NPAS structure. OB1 contains five additional acceptor subsites (+2, +3, +4, +5, and +6), and the

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nonreducing end of the maltoheptose (G7) molecule bound to OB1 contacts the-1 subsite. SB2 is close to the active site and contains four amino acids belonging to the B′-domain. OB1 and OB2 also contain various amino acids belonging to the B′-domain. Therefore, the B′-domain in ASase includes acceptor binding sites [17] and, unlike other G13 family enzymes, both the B and B′-domains are thought to play important roles in the polymerization activity of ASase [18].

In previous studies, NPAS has been engineered to improve its biochemical properties such as polymerization activity and acceptor specificity, based on the identified interactions between acceptor molecules and the NPAS molecule [19–23]. However, despite the demonstration of the molecular evolution of NPAS, few studies have attempted to address such potential for other ASases.

In this study, the biochemical properties of four ASases (ACAS, AMAS, DGAS, and NPAS) were compared. Based on these results, DGAS and NPAS were selected for domain-swapping mutagenesis as introduced by overlap extension polymerase chain reaction (OE-PCR). We constructed six chimeric ASases and investigated their properties through biochemical analysis. Among these, one chimeric ASase showed unexpected ASase properties by altering its polymerization activity not to produce longer α-1,4-glucans.

2. Materials and methods

2.1. Chemicals and enzymes

Sucrose was obtained from Duchefa Biochemistry (Haarlem, Netherlands). DNA-modifying enzymes, including restriction endonucleases T4 DNA ligase and PrimeSTAR HS DNA polymerase, were purchased from New England Biolabs (Beverly, MA, USA) or Takara (Kyoto, Japan). A HisTrap™ HP affinity column was obtained from GE Healthcare (Uppsala, Sweden) and used for the purification of 6 × histidines (6 × His)-tagged recombinant proteins. All other chemicals used in this study were of analytical reagent grade from Sigma-Aldrich Chemical Co (St. Louis, MO, USA).

2.2. Bacterial strains and culture conditions

Escherichia coli DH10B [F⁻ *end*A1 *rec*A1 *gal*U *gal*K *nup*G *rps*L Δ(*lac*)X74 Φ80d*lac*ZΔM15 *ara*D139 Δ(*ara*, *leu*) 7697 *mrc*A Δ(*mrr hsd*RMS *mcr*BC) λ⁻] was used as the host for typical DNA manipulation and transformation. *E. coli* BL21 [F–, *omp*T, *hsd*SB(rB-, mB-), *dcm*, *gal*, (DE3)] (Novagen, Darmstadt, Germany) was employed for the expression of inducible pET-21a(+) based vectors. Recombinant *E. coli* strains were grown in Luria-Bertani (LB) medium containing 1% (w/v) Bactotryptone, 0.5% (w/v) yeast extract, and 0.5% (w/v) NaCl, supplemented with ampicillin (100 μg/mL) at 37 °C.

2.3. Construction of chimeric genes by overlap extension polymerase chain reaction

The genes encoding the *dgas* (ASase from *D. geothermalis*, accession number ABF44874) and *npas* (ASase from *N. polysaccharea*, accession number AJ011781) were amplified from pGEX-DGAS [6] and pRSET-NPAS [9] by PCR using the oligonucleotide DGAS-F/DGAS-R and NPAS-F/NPAS-R, respectively (Table 1). PCR conditions were described previously [6,9]. Both PCR products were cloned into the pET-21a(+) vector (Novagen) using *Nde*I/*Xho*I and *Eco*RI/*Xho*I restriction sites, respectively. The final expression plasmids, designated pETDGAS and pETNPAS, were transformed into the host *E. coli* BL21 (DE3) for recombinant protein production.

Ten segments from the *dgas* and *npas* genes were amplified by PCR using the plasmids pETDGAS and pETNPAS (Fig. S1). The resulting PCR products were directly cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA) followed by DNA sequencing analysis to confirm the absence of PCR-introduced errors. The PCR amplification was performed on a TaKaRa PCR Thermal Cycler Dice™ cycler (Takara). PCR condition for each PCR reaction were as follows: 50 ng template DNA, 1 mM of each primer, 0.25 mM of each dNTP, 5 × PrimeSTAR DNA polymerase buffer, and 1 U PrimeSTAR DNA polymerase (Takara). PrimeSTAR DNA polymerase was used to obtain low error PCR products. 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 a last elongation step for 7 min at 72 °C.

Each of the gene segments was gel-purified using a separate agarose gel by using the Axygen Gel Extraction Kit (Axygen Scientific Inc., Union City, CA, USA). The various segment combinations of DGAS and NPAS were mixed and shuffled in equimolar ratios. Each of domain segment replacement (DSR) fragments was amplified using the purified segment combinations listed in Table S1. Overlap extension PCR amplification was carried out in following conditions: approximately 50 ng of purified segment DNAs, 1 mM of each external primer, 0.25 mM of each dNTP, 5 × PrimeSTAR DNA polymerase buffer, and 1 U PrimeSTAR DNA polymerase (Takara). The PCR conditions were the same as above.

Chimeric PCR products were gel-purified and digested with each of the restriction endonucleases. In order to prepare chimeric genes, which are substituted in the B and B' domains, respectively, the digested chimeric PCR products were exchanged and ligated into the parental genes (pre-digested with the same restriction enzyme, Fig. S2). The pET-21a(+) vectors digested with *Eco*RI/*Xho*I for NPAS and *Nde*I/*Xho*I for the DGAS chimeric gene, respectively, were used to construct the inducible expression system. The constructed expression vectors, pETNPAS-B, pETNPAS-B', pETNPAS-BB', pETDGAS-B, pETDGAS-B', and pETDGAS-BB' were successfully generated and the six-histidine tag was attached to the C-terminal residues in frame.

2.4. Expression and purification of recombinant enzymes

The constructed expression vectors were transformed into *E. coli* BL21(DE3) cells for efficient heterologous protein expression. The induction was started by growing the recombinant *E. coli* BL21(DE3) harboring pETNPAS, pETNPAS-B, pETNPAS-B', pETNPAS-BB', pETDGAS, pETDGAS-B, pETDGAS-B', pETDGAS-BB', pETACAS [8], or pETAMAS [7], respectively, in 1 L of LB medium supplemented with 0.1 mg/mL of ampicillin at 37 °C with agitation until the optical density reached 0.5 to 0.6 at 600 nm. Then IPTG was added to a final concentration of 1 mM followed by a 3 h growth period to induce the expression of the chimeric gene.

The cells were harvested by centrifugation (7,000 × *g* for 20 min at 4 °C) and washed with a binding buffer containing 20 mM Tris–HCl, 500 mM NaCl, and 20 mM imidazole (pH 7.0). The bacterial pellet was resuspended in binding buffer and disrupted by sonication (Sonifier 450, Branson, Danbury, CT, USA; output 4, 6 × 10s, constant duty) in an ice bath. The supernatant acquired by centrifugation (10,000 × *g* for 10 min at 4 °C) was used as a starting material to obtain recombinant proteins. Purification of recombinant chimeric enzymes was performed using a fast performance liquid chromatography (FPLC) system with a HisTrap HP column (GE Healthcare). The cell lysate was loaded into the HP column and eluted with elution buffer [20 mM Tris–HCl, 500 mM NaCl, and 500 mM imidazole (pH 7.0)] at a flow rate of 1.0 mL/min. All fractions were collected and confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 10% (w/v) acrylamide gel. The protein concentration was determined using the BCA™ protein assay kit (Pierce Biotechnology Inc., Rockford, IL, USA) with bovine serum albumin (BSA) as a standard.