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Biochemical characterization of a highly thermostable amylosucrase from *Truepera radiovictrix* DSM 17093

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ABSTRACT

In this study, a novel recombinant amylosucrase from *Truepera radiovictrix* DSM 17093 was characterized and found to produce α-glucans from sucrose. The enzyme showed maximum total and transglucosylation activities at pH 7.5 and maximum hydrolysis activity at pH 5.5. The optimum temperature for total, transglucosylation, and hydrolysis activities were determined to be 45, 45, and 50 °C, respectively. When the conversion of 100 mM sucrose was catalyzed at 35, 45, and 55 °C for 24 h, TR-ASase produced α-(1,4) glucans with average DPs of 59, 45, and 37, respectively. TR-ASase displayed more than 90% of its original activity after incubation at 55 °C for 5 h, which was much higher than that of all other reported ASases. Melting temperature determination and homology modeling were also adopted to analyze the extreme thermostability of this enzyme, TR-ASase.

1. Introduction

Amylosucrase (ASase, sucrose: 1,4-α-D-glucan 4-α-D-glucosyltransferase, EC 2.4.1.4), one of the enzymes in the glycoside-hydrolases sub-family 13_4, catalyzes the transfer of α-D-glucosyl from sucrose to the non-reducing end of an α-glucan chain with the concomitant release of D-fructose from sucrose, leading to the generation of an insoluble amylose-type α-glucan polymer with only α-(1,4) linkages. It displays sucrose hydrolysis activity when reacting with water as a glucosyl acceptor substrate. ASase shows great potential for application in starch modification. ASase can catalyze the elongation of external amylopectin chains with sucrose as a glucosyl donor substrate and increase the proportion of long chains in starch molecules, leading to high contents of slowly digestible and resistant starches with low digestion properties and low glycemic response [1–4]. Like some other glycotransferases, ASase may catalyze transglycosylation from sucrose to a wide range of oxy-compounds as acceptor molecules and produce the corresponding bioactive α-glucosides, including arbutin α-glucoside [5], (+)-catechin α-glycosides [6], dihydrochalcone glucosides [7], α-D-glucosyl glycerol [8], salicin glycosides [9], and rutin derivatives [10].

ASase has recently attracted increasing attention due to its great potential for the production of some functional carbohydrates. Generally, in the presence of sucrose as a sole substrate, ASase uniquely produces a certain amount of sucrose isomers, especially turanose and trehalulose, because of the transfer of an α-D-glucosyl moiety from sucrose to the released D-fructose molecule [11]. As a structural isomer of sucrose, turanose has been considered as an ideal sucrose substitute for applications in the food industry. Wang et al. first optimized the of highly efficient production of turanose by ASase, and the turanose yield reached 56.2% in the presence of sucrose as the sole substrate [12]. Recently, Park et al. further improved the turanose yield to 73.7% by using extrinsic D-fructose as a reaction modulator [13]. In addition, amylose-like α-(1,4)-glucan, biosynthesized from sucrose by ASase, has been supposed as a more effective substrate than natural starch molecules for the production of cycloamyloses (CA) and cyclodextrins (CD), which both have great potential in applications in the food, pharmaceutical, and chemical industries. Kim et al. and Koh et al. reported the efficient conversion of sucrose to CAs and CDs by the concomitant dual enzyme reaction of ASase coupled with 4-α-glucanotransferase [14] and cyclodextrin glucanotransferase [15], respectively. The linear α-(1,4)-glucan produced by ASase has also been used as an ideal substrate for trehalose production by a maltooligosyl trehalose synthase-trehalose hydrolase system, and it provides a feasible approach to the direct conversion of sucrose to trehalose, which is of great interest in many industrial areas [16].

Most of the characterized ASases are from mesophilic microorganisms, including *Alteromonas macleodii* KCTC 2957 (AM-ASase) [17], *Arthrobacter chlorophenolicus* A6 (AC-ASase) [18], *Cellulomonas carbonis* T26 (CC-ASase) [19], *Methylobacillus flagellatus* KT (MF-ASase) [8], *Methylomicrobium alcaliphilum* 20Z (MA-ASase) [20], *Neisseria perflava* (NPr-ASase) [21], *Neisseria polysaccharea* ATCC 43768 (NPo-ASase) [22], and *Synechococcus* sp. PCC7002 (SS-ASase) [23], and these ASases generally do not show high thermostability. NPo-ASase, the earliest found and the most extensively studied ASase, has been widely used for starch modification [2,4,24] and the enzymatic production of various functional products, including turanose [12], CAs [14], CDs [15], and many bioactive α-glucosides [7,9]. However, it is suggested that the low thermostability of ASase might limit its industrial application [25].

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Directed evolution and high-throughput screening have been used to effectively improve NPo-ASase thermostability [26,27].

High thermostability is commonly considered an important factor in evaluating the feasibility of the industrial application of carbohydrate conversion-related enzymes, such as α-amylase [28], branching enzymes [29], cyclodextrin glucanotransferase [15], and 4-α-glucanotransferase [14]. Thermophilic and hyperthermophilic microorganisms are usually important sources for obtaining the abovementioned thermostable enzymes [29]. ASase from *Deinococcus geothermalis* (DG-ASase), a thermophile, has been identified to be relatively thermostable [25]. DG-ASase has much higher thermostability than the others enzymes and shows half-lives of 28.1 and 6.8 h at 50 and 55 °C, respectively [25]. DG-ASase has been used for amylose microparticles [30–32] and many bioactive α-glucosides [5,6,9,33].

In this work, a hypothetical thermostable ASase was sought based on a protein sequence BLAST search focused on enzymes from thermophilic microorganisms. *Truepera radiovictrix* DSM 17093, a moderately thermophilic bacterium, was used. A recombinant *T. radiovictrix* ASase (TR-ASase), heterologously expressed in *Escherichia coli*, was purified and identified and exhibited the highest thermostability of all reported ASases.

2. Materials and methods

2.1. Database search and amino acid sequence analysis

To obtain putative ASases, a protein sequence BLAST search was carried out against a public database using the NCBI BLAST tool and querying the protein sequence of the previously reported protein DG-ASase (GenBank accession No. ABF44874.1). The protein sequence alignment was conducted using ESPript software.

2.2. Gene cloning and heterologous expression

The full nucleotide sequence of a putative ASase-encoding gene (locus_tag: TRAD_RS05145) from *T. radiovictrix* DSM 17093 was commercially synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The synthesized gene was linked to a sequence encoding 6 × histidine-tag at the 3′-terminus and inserted into the *Nde*I and *Xho*I sites of a pET-22b(+) vector, giving rise to a recombinant plasmid termed pET-TR-ASase. The recombinant plasmid was transformed into *E. coli* BL21(DE3) for expression. The recombinant cells were first grown at 37 °C in Luria-Bertani (LB) broth supplemented with 100 μg/mL^{-1} ampicillin until the optical density at 600 nm reached 0.6. Heterologous expression of TR-ASase was then induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and the culture was grown for another 6 h at 28 °C.

2.3. Affinity chromatography purification of the recombinant TR-ASase

Cells were harvested from culture broth by centrifugation at 6000*g* for 20 min at 4 °C and resuspended in 50 mM Tris-HCl buffer (pH 7.5) containing 100 mM NaCl. The resuspended cells were disrupted on ice by ultrasonication for 15 min using a Vibra-Cell 72,405 Sonicator (Bioblock, Illkirch, France). The process included pulsing on for 1 s and off for 2 s. The cell debris was removed by centrifugation at 18,000*g* for 30 min at 4 °C. The supernatant was filtered through a 0.45 μm filter and then loaded onto a His-Trap HP chromatography column (Amersham Biosciences, Uppsala, Sweden). The column was equilibrated with 50 mM sodium phosphate buffer (pH 7.5) containing 500 mM NaCl and washed extensively with the same buffer containing 50 mM imidazole, and the protein was then eluted with a linear gradient from 50 to 500 mM imidazole in the same buffer. The active fractions were collected and dialyzed against 50 mM sodium phosphate buffer (pH 7.0). All the purification steps were conducted at 4 °C.

2.4. Molecular mass analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a 5% stacking gel and a 12% separating gel was carried out to measure the subunit molecular mass, and the gels were stained by Coomassie Brilliant Blue R250. Gel filtration was performed to estimate the total molecular mass of the native enzyme using a TSKgel G2000SWxl column (Tosoh Bioscience LLC, Minato-ku, Tokyo, Japan). Phosphate buffer (100 mM, pH 6.7) containing 100 mM Na_2SO_4 and 0.05% (w/v) NaN_3 was used as the mobile phase at a flow rate of 1 mL/min, and the absorbance of the protein was detected at 280 nm.

2.5. Enzyme assay

Amylosucrase activity was assayed using sucrose (100 mM) as a sole substrate at pH 7.5 and 45 °C for 4 h. Total activity and hydrolytic activity were determined by calculating the release of fructose and glucose, respectively. Transglycosylation activity was defined as the total activity minus the hydrolytic activity. One unit of total activity and hydrolytic activity corresponded to the amount of enzyme that catalyzed the release of 1 μmol of fructose and glucose per min, respectively. Unless stated otherwise, total activity was used to describe the enzyme activity in this work.

2.6. Effect of pH and temperature

The effect of pH on enzyme activity was studied within the range of pH 4.5 to 8.5 at 45 °C (50 mM acetate buffer for pH values 4.5 to 6.0, 50 mM sodium phosphate buffer for pH 6.0 to 7.5, and 50 mM Tris-HCl for pH 7.5 to 8.5). The effect of temperature on the activity was investigated between 40 and 60 °C at pH 7.5. The effect of temperature on the stability was assessed by incubation of the purified TR-ASase in the absence of substrate at 55, 60, and 65 °C. The residual activity was measured under standard assay conditions at certain time intervals.

Differential scanning calorimetry (DSC, TA Instruments Nano DSC, New Castle, PA) was used to determine the melting temperature (T_m) of the purified enzyme. After being redialyzed against sodium phosphate buffer overnight and degassed under vacuum (635 mmHg) for 10 min, the enzyme solution was loaded into the DSC cell. DSC experiments (heating range: 25 to 100 °C, temperature ramp: 1 °C/min, pressure: 3 atm) were performed and DSC data were analyzed by TA Instruments NanoAnalyze software with the dialyzed buffer served as the corresponding reference.

2.7. Molecular modeling

The homology structural model was constructed using the SWISS-MODEL server (https://swissmodel.expasy.org/) and the experimentally determined structure of DG-ASase (PDB ID: 3UCQ) as a template. The structure was validated by the Structure Analysis and Verification Server (http://services.mbi.ucla.edu/SAVES/) and presented with the PyMOL Molecular Graphics System.

2.8. Analysis of soluble reaction products

After the conversion of 100 mM sucrose was catalyzed by TR-ASase at pH 7.5 and 45 °C for 24 h, the reaction solution was centrifuged at 13,000*g* for 20 min to remove the insoluble products. The soluble products were analyzed by high-performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD). The HPAEC-PAD system (Dionex DX 600, Dionex Corp., Sunnyvale, CA) was equipped with an ED 50 electrochemical detector. Short oligosaccharides and relatively long polysaccharides were analyzed by using Dionex CarboPac PA1 and PA200 columns, respectively. Eluents A (100 mM NaOH in water) and B (500 mM sodium acetate in eluent B) were used. Filtered samples were eluted using a linear gradient ranging from 100% eluent A to 60% eluent B. The flow rate of the mobile phase was maintained at 1 mL/min.