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A recombinant levansucrase from *Bacillus licheniformis* 8-37-0-1 catalyzes versatile transfructosylation reactions

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ABSTRACT

This work disclosed the broad transglycosylation capability of the levansucrase from *Bacillus licheniformis* 8-37-0-1 for the first time. The levansucrase was firstly purified from the strain 8-37-0-1 and found to be a monomer of ~51 kDa with KETQDYKKSY as the *N*-terminus. Then, the gene encoding the enzyme was cloned and it contained an ORF of 1449 nucleotides, encoding a 482 amino-acid protein with a predicted 29 amino-acid signal peptide. The deduced mature protein without the signal showed the same *N*-terminus to the purified enzyme. The mature enzyme was subsequently expressed in *Escherichia coli*. The recombinant enzyme showed similar biochemical properties to the native one. It produced maximal yield of 7.1 mg/mL levan (M_r 9.6×10^6) from 0.8 M sucrose (pH 6.5) at 40 °C for 24 h *in vitro*. When using sucrose as the donor, the enzyme displayed a wide range of acceptor specificity and was able to transfer fructosyl to a series of sugar acceptors including hexose, pentose, β- or α-disaccharides, along with the difficult branched alcohols that have not been investigated before. Chemical structures of the resultant products were analyzed by MS and NMR spectra.

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1. Introduction

Levansucrases (EC 2.4.1.10) catalyze fructosyl transfer following the mechanism of non-Leloir glycosyltransferases with sucrose as the main non-activated donor substrate. Such non-Leloir enzymes have great biotechnological potential, since they do not require expensively activated donors such as the nucleotide phosphosugars that are necessary for Leloir enzymes. The Gibbs binding energy released from hydrolysis of sucrose is available for saccharide formation [1].

Levansucrases are widely distributed in nature, especially abundant in gram-negative and gram-positive bacteria. They are of particular interest for the formation of levan, a β-(2→6)-linked fructose polymer with small amount of β-(2→1)-linked branch chains [2–4]. The degree of polymerization and type of branching are various depending on enzyme sources. These polymers possess wonderful physiological and biochemical characteristics and thus have broad applications. They can be widely used in food and non-food industries as viscosifier, stabilizer, emulsifier, gelling, or water-binding agent. Also, they have potential pharmaceutical applications owing to diverse bioactivities, such as antiviral, antitumor and immunostimulating activities [5–8].

In addition to levan, fructooligosaccharides (FOS) are also produced by some levansucrases *via* oligomerization from sucrose. Such kind of oligomers are described as healthful prebiotics, which exhibit low caloric values, decrease levels of lipids and cholesterol, help gut absorption of ions, and stimulate the *bifidobacteria* growth in the human colon [9]. Using sucrose as glycosyl donor, levansucrases can also catalyze fructosyl transfer to a wide range of acceptors including alcohols and mono- or oligosaccharides. The different sugar residues (*i.e.* galactose and xylose) that cap fructooligosaccharides may alter prebiotic and biochemical properties [10]. Some of the hetero-oligosaccharides have been found applications as sweeteners and as prebiotics [11]. Despite the promising prospect of developing fructosyl compounds with novel extended functions, the reported levansucrases are mostly focused on the formation of levan and FOS, while only a few of them have been fully characterized with respect to their acceptor specificity for transglycosylation [12–16].

In the previous work, a strain of *Bacillus licheniformis* 8-37-0-1 with high level of levan production (41.7 g/L) was isolated from the soil [17]. The levan product was subsequently purified from the fermentation broth and identified to be a novel polysaccharide, which contained a β-(2→6)-linked backbone with a single β-D-fructose at the C-1 position every seven residues, on average, along the main chain. Preliminary *in vitro* tests revealed it could significantly stimulate the proliferation of spleen lymphocyte [18].

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In this work, the levansucrase was purified from *B. licheniformis* 8-37-0-1, and the gene of the enzyme was cloned and over-expressed in *Escherichia coli*. The resulting recombinant enzyme was found to possess similar biochemical properties to the native one and thus was used in the synthesis of levan *in vitro*. Also, the enzyme was found to catalyze oligomerization reactions. It could efficiently transfer fructosyl to a lot of saccharides as well as novel branched alcohol acceptors. This is the first report of the powerful transglycosylation ability of the levansucrase from *B. licheniformis* 8-37-0-1. The excellent characteristics endowed the enzyme with a high capacity for obtaining novel fructosyl compounds and made it an alternative to the current synthetic enzyme sources.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture conditions

B. licheniformis 8-37-0-1 was cultured at 37 °C for 24 h in the medium (pH 7.0) containing 30 g/L sucrose, 1.0 g/L beef extract, 0.5 g/L $(NH_4)_2SO_4$, 2.5 g/L $K_2HPO_4{\cdot}3H_2O$, 2.5 g/L KH_2PO_4, 1.0 g/L NaCl, 0.2 g/L $MgSO_4{\cdot}7H_2O$, and 0.001 g/L $FeSO_4{\cdot}7H_2O$. *E. coli* strains DH5α and BL21 (DE3) were grown at 37 °C in LB medium. The pET-21b (+) plasmid (Novagen, US) was used for the construction of the expression vector with the His tag. The medium for the *E. coli* cells containing the pET-21b (+) plasmid was supplemented with ampicillin (100 μg/mL).

2.2. Enzyme and protein assays

The activity of levansucrase was measured by addition of enzyme solution (10 μL) to 40 μL of sucrose (finally at 0.1 M) in 50 mM phosphate buffer (pH 6.0). The reaction was performed at 37 °C for 60 min and then stopped by heating at 100 °C for 10 min. The resulting mixture was centrifuged and the glucose content in the mixture was analyzed using the glucose oxidase kit (Biosino Biotechnology and Science Inc., China). One unit of enzyme activity (U) is defined as the amount of enzyme that produces 1 μmol glucose per minute under the assay conditions. The concentrations of protein were measured according to Bradford method using BSA as the standard [19]. All enzyme and protein determinations were performed in triplicate.

2.3. Enzyme purification

All the procedures described below were performed at 4 °C. The culture of *B. licheniformis* 8-37-0-1 was centrifuged at 12,000 rpm for 5 min. The resultant supernatant was precipitated with ammonium sulfate at 80% saturation, followed by desalting. The sample was subsequently applied to a 1.1 × 20-cm DEAE Sepharose Fast Flow column (GE Healthcare, US) which had been pre-equilibrated with 50 mM phosphate buffer (pH 7.5). Then it was eluted by NaCl solution (pH 7.5) at concentration gradients from 0 to 0.4 M. The collected enzyme fragments were concentrated through centrifugal filters (Millipore, Germany) with a 30-kDa molecular mass cutoff.

2.4. Molecular mass determination

The molecular mass of levansucrase was determined by SDS polyacrylamide gel electrophoresis (SDS-PAGE) as well as gel filtration chromatography. Proteins in the polyacrylamide gel were visualized by Coomassie brilliant blue R-250 staining. Gel filtration chromatography was performed *via* a Superdex 200 (10 × 300 mm) column (GE Healthcare, US) pre-equilibrated with 150 mM NaCl in 50 mM phosphate buffer. Samples were eluted at a flow rate of 0.3 mL/min at 4 °C. The elution patterns were compared with those of the standard proteins, including ovalbumin (44 kDa), conalbumin (75 kDa), aldolase (158 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa).

2.5. Gene cloning and heterogenous expression

Primers for the gene cloning were designed based on the sequence of the levansucrase from *B. licheniformis* DSM 13 (GenBank No. AE017333). The forward and reverse primers designed for the entire gene were 5′-CAGGTCGACATGAACACATCAAAAACATTGC-3′ and 5′-CGACTCGAGTTTGTTTACCGTTAGTTGTCC-3′ (*Sal* I and *Xho* I restriction sites are underlined), respectively. As for the gene without the signal-encoding sequence, the forward primer was 5′-CAGCATATGAAAGAAACGCAGGATTACAAG-3′ (*Nde* I site is underlined). The PCR reactions were performed in the presence of TaKaRa LA Taq polymerase, following the procedures including 5 min at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 55 °C, 2 min at 72 °C, and a final 5 min at 72 °C. PCR products were purified and sequenced.

For enzyme expression, the purified PCR products were subsequently digested by restriction enzymes, ligated onto the pET-21b (+) vector, and transformed into *E. coli* BL21 (DE3). The correct transformant was grown in LB medium at 37 °C, and the enzyme was induced by adding isopropyl-1-thio-β-D-galactoside (IPTG) when the cell density reached 0.6–1.0 at 600 nm. After continuous cultivation for three to 5 h, cells were harvested and disrupted by ultrasonic treatment. The lysate was centrifuged and the enzyme was purified from the suspension by Ni^{2+} chelation chromatography (Qiagen, Germany).

2.6. Characterization of native and recombinant levansucrase

Kinetic constants of native and recombinant enzymes were estimated by Lineweaver–Burk double reciprocal plots. Various concentrations of sucrose (0.1–1 M) were used to determine kinetic constants. Reactions were performed under assay conditions as described above. The effect of pH on the activity of the enzyme was determined by incubating the enzyme with 0.1 M sucrose in broad-range buffers containing 6.008 g/L citric acid, 3.893 g/L KH_2PO4, 1.769 g/L boric acid and 5.266 g/L barbitone and using NaOH to adjust the pH from 4.0 to 9.0. The effect of temperature was assayed at 20 °C to 65 °C. All assays were performed in triplicate.

2.7. Levan synthesis and analysis

Levan synthesis was carried out with the recombinant mature levansucrase in the presence of sucrose as substrate. The effects of sucrose concentrations were determined by incubating enzymes (50 U/mL) with sucrose solutions ranging from 0.1 M to 1 M in the pH 8.0 phosphate buffer at 37 °C for 24 h. The influence of the reaction time was assayed in 0.8 M sucrose at 37 °C. Aliquots were serially removed at 12 h intervals within 60 h period. The effects of temperature were assayed at 25, 30, 35, 40, 45, and 50 °C for 24 h. The impacts of pH on levan synthesis were performed in broad-range buffers with pH values ranging from 4.0 to 9.0 at 40 °C for 24 h. All the reactions were stopped by boiling at 100 °C for 10 min. Then the mixtures were centrifuged at 12,000 rpm for 5 min. Levan in the suspension was precipitated by 75% ethanol and quantified by the phenol-sulfuric acid method [17]. In the method, the polysaccharide was hydrolyzed by the concentrated sulfuric acid and released the monosaccharides that were quickly dehydrated, reacted with phenol and converted into colorimetric compounds. The absorbance of the compounds at certain wave length showed linear relationship with the sugar content in some range. A

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