International Journal of Biological Macromolecules 92 (2016) 723–730

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

# Identification of a novel DFA I-producing inulin fructotransferase from *Streptomyces davawensis*

Yingying Zhu[a], Shuhuai Yu[a], Danyang Huang[a], Tao Zhang[a], Bo Jiang[a,b], Wanmeng Mu[a,b,*]

[a] State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu, 214122, China
[b] Synergetic Innovation Center of Food Safety and Nutrition, Jiangnan Universtiy, Wuxi, Jiangsu, 214122, China

ARTICLE INFO

*Article history:*
Received 17 January 2016
Received in revised form 23 June 2016
Accepted 27 July 2016
Available online 28 July 2016

*Keywords:*
Di-D-fructofuranose 1,2′:2,1′ dianhydride (DFA I)
Inulin
Inulin fructotransferase
Homology modelling
Molecular docking

ABSTRACT

In this work, a novel gene encoding DFA I-forming inulin fructotransferase (IFTase) from *Streptomyces davawensis* SK39.001 was cloned and expressed in *Escherichia coli*. The enzyme was purified, identified, and characterized. The results showed that this IFTase (DFA I-forming) is a trimer (molecular weight of 125 KDa) consisting of three identical subunits (the molecular weight as assayed by SDS-PAGE was approximately 40 KDa). At pH 5.5 and 40 °C, the maximum specific activity (approximately 100 U $mg^{-1}$) was achieved. Moreover, the enzyme was stable up to 70 °C. $K_m$ and $V_{max}$ were 2.89 ± 0.2 mM and 1.94 ± 0.9 mM $min^{-1}$, respectively. For exploring putative active sites and probable catalytic mechanisms, homology modelling and molecular docking methods after site-directed mutagenesis were applied to IFTase (DFA I-forming). The results revealed that D183 and E194 were potential catalytic residues of the purified enzyme.



## 1. Introduction

Two basic fructans, inulin and levan, are β-(2,1)- and β-(2,6)-linked fructose polymers terminated with sucrose residues, respectively. Due to extensive sources and a relatively low price, inulin can be widely biologically converted into various high-value products commercially, such as inulooligosaccharides (IOS), single-cell protein and difructose dianhydrides (DFAs) [1]. The production of these substances requires several inulin-decomposing enzymes, including *endo*-inulinase [2], *exo*-inulinase [3], and inulin fructotransferase (IFTase). In recent years, di-D-fructofuranose 1,2′:2,3′ dianhydride (DFA III) and its biosynthesized enzyme, IFTase (DFA III-forming), have been attracting attention because of the potential physiological functions of DFA III. To date, more than 10 microorganisms have been identified as having the ability to produce IFTases (DFA III-forming) [4]. One of these microorganisms, *Arthrobacter* sp. H65-7, has a relatively high productivity of IFTase (DFA III-forming) and extracellular enzyme activity (90 U $ml^{-1}$) [5]. Since 2004, the Shimizu Factory (Tokachi District, Hokkaido, Japan) of Nippon Beet Sugar Mfg. Co., Ltd. has industrially manufactured DFA III using commercially available *Arthrobacter* sp. H65-7 IFTase (DFA III-forming) [6]. Moreover, in Japan, DFA III-containing commodities have been on the market since 2004 and in drugstores and convenience stores since 2011 [7].

However, by comparison, little literature has focused on the physiological functions and practical application of di-D-fructofuranose 1,2′:2,1′ dianhydride (DFA I). Although DFA I is a potential new type of low calorie sweetener [7], it is not clear whether it has prebiotic properties as does DFA III. Thus far, the only functional aspect known for DFA I is its half-sweetness of sucrose [7]. To some extent, the lower availability of DFA I might limit the physiological functional investigation of DFA I. Bioconversion of inulin is an effective pathway to obtain DFA I; however, reports are much less frequent [8]. Previously, six studies were conducted on the biosynthesis of DFA I using IFTase (DFI-forming) [4], involving six microorganisms. These microorganisms are *A. globiformis* S14-3 [9], *Streptomyces* sp. MCI-2524 [10], *Arthrobacter* sp. MCI2493 [11], *A. ureafaciens* A51-1 [12], *A. pascens* a62-1 [13], and *Arthrobacter* sp. B69-5 [14]. To date, there are only two reports of the gene cloning and heterogeneous expression of IFTase (DFA I-forming), including IFTases (DFA I-forming) originating from *Clostridum clostridioforme* AGR2157 [15] and *Arthrobacter globiformis* S14-3 [16]. The majority of the above seven microorganisms (five of seven) are classified as *Arthrobacter*; therefore, the exploration of novel IFTase (DFA I-

* Corresponding author at: State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu, 214122, China.
*E-mail address:* wmmu@jiangnan.edu.cn (W. Mu).

http://dx.doi.org/10.1016/j.ijbiomac.2016.07.092
0141-8130/

forming)-producing microorganisms will be helpful for realizing mass production and further functional investigations of DFA I.

In our previous work, we identified a new IFTase (DFA I-forming) named *Cc*IFTase from *C. clostridioforme* AGR2157. This enzyme exhibited promising properties (especially for its high thermostability) for the production of DFA I [15]. In this work, we identified a novel IFTase (DFA I-forming) from *Streptomyces davawensis* SK39.001. Herein, routine investigations of gene cloning, heterogeneous expression, purification and characterization of IFTase (DFA I-forming) are described. Furthermore, to explore putative active sites and probable catalytic mechanism of IFTase (DFA I-forming), including the enzyme (purified in this work) and *Cc*IFTase (investigated in our previous work), homology modelling and molecular docking methods were used. The computational analyses provided a way to excavate potential structure information and these studies may be helpful for further investigation of the catalytic mechanism of IFTase (DFA I-forming) through crystal structure. For the convenience of description, the purified enzyme from *S. davawensis* SK39.001 in this work was abbreviated as *Sd*IFTase.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Inulin (Orafti HP, from chicory, molecular weight assumed as 5000 Da) was purchased from BENEO-Orafti NV (Tienen, Belgium). The reagents 1-kestose ($GF_2$), nystose ($GF_3$), and fructofuranosyl nystose ($GF_4$) were from Wako Pure Chemical Industries (Osaka, Japan). The agents for gene cloning were obtained from Sangon Biotech Co., Ltd. (Shanghai, China). Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was purchased from Sigma (St. Louis, Mo, USA). The resin for the $Ni^{2+}$-Chelating affinity chromatography was from GE (Uppsala, Sweden). Electrophoretic reagents were obtained from Bio-Rad (Hercules, CA, USA). Ion-exchange resin DTF was purchased from Jiangsu Suqing Water Treatment Engineering Group Co., Ltd. (Wuxi, China). All chemicals used for enzyme assays and characterization were at least of analytic grade and were obtained from Sigma (St. Louis, MO, USA) or Sinopharm Chemical Reagent (Shanghai, China).

### 2.2. Cultivation of microorganisms

The *S. davawensis* SK39.001 was previously isolated from soil by our laboratory and kept at the China Center for Type Culture Collection (CCTCC) under the accession number M 2015352. To activate the species, *S. davawensis* SK39.001 was first cultivated in a 250 ml flask containing 100 ml of seed medium with a rotary shaker at 200 rpm and 28 °C for 16 h. The seed medium was composed of yeast extract 2.0 g $l^{-1}$, soluble starch 10 g $l^{-1}$, and agar 15 g $l^{-1}$, with a pH of 7.3. Subsequently, a two percent of seed culture was inoculated into the fermentation culture (200 ml in a 500 ml flask) and was shaken at 200 rpm and 28 °C for 72 h. The fermentation medium contained yeast extract 2.0 g $l^{-1}$, inulin 10 g $l^{-1}$, and agar 15 g $l^{-1}$, at a pH of 7.3.

### 2.3. Gene cloning and heterogeneous expression

After cultivation, the cells of *S. davawensis* SK39.001 were harvested by centrifugation (10,000 × *g*, 20 min). The chromosomal DNA acting as a template for polymerase chain reaction (PCR) was prepared from these cells using a Genomic DNA Isolation Kit (Sangon, Shanghai, China) according to the manufacturer's protocol. To obtain the target IFTase gene from chromosomal DNA of *S. davawensis* SK39.001, mixed oligonucleotide primers, including the sense primer (5′- ATGGCCACTGTCTACGACGTCA -3′) and the antisense primer (5′- TCAGGGTGTGGCCACGAGC -3′), were synthesized chemically. These primers were designed according to a putative IFTase gene (gene locus_tag: BN159_0187; protein ID: CCK24566) in *S. davawensis* JCM 4913, whose complete genome sequence has been released in GenBank (NCBI accession number: HE971709.1). The routine PCR process was performed using Taq Plus DNA polymerase for 35 cycles, consisting of 94 °C for 30 s, 57 °C for 1 min, and 72 °C for 1 min, followed by a final extension step at 72 °C for 10 min. Subsequently, the amplified DNA fragment of 1179 bp was extracted using the SanPrep column DNA gel extraction kit and was sequenced by Sangon Biological Engineering Technology and Service (Shanghai, China). For the subcloning and expression of this DNA fragment, the pET-22b(+) vector with *Nde*I and *Xho*I sites was used. The recombinant vector was introduced into *Escherichia coli* BL21(DE3) (genotype: B $F^-$ *dcm ompT hsdS* ($rB^-$ $mB^-$) *gal*λ(DE3) obtained from Sangon Biological Engineering Technology and Services (Shanghai, China)). When the optical cell density of the transformed *Escherichia coli* BL21(DE3) culture at 600 nm ($OD_{600}$) reached 0.6, the overexpression of IFTase was induced by adding IPTG at a final concentration of 1 mM at 28 °C for 72 h.

### 2.4. Purification and estimation of enzyme molecular weight

After overexpression for 72 h, the cells were centrifuged at 10,000 × *g* (4 °C, 20 min), and the supernatant was filtered through a 0.22 μm filter and applied to a $Ni^{2+}$-chelating Sepharose Fast Flow column (GE Healthcare, Uppsala, Sweden) equilibrated with binding buffer (50 mM sodium phosphate buffer, 500 mM NaCl, pH 7.5). Washing buffer (50 mM sodium phosphate buffer, 500 mM NaCl, 50 mM imidazole, pH 7.5) and elution buffer (50 mM sodium phosphate buffer, 500 mM NaCl, 500 mM imidazole, pH 7.5) were applied to remove unbound protein and obtain the target enzyme, respectively. Finally, the eluate was dialyzed against sample buffer (50 mM citrate buffer, pH 5.5) for 24 h to eliminate imidazole. This dialytic solution was used as the purified enzyme solution for further studies.

Based on the method of Bradford [17] the protein concentration was measured with bovine serum albumin as a standard. For estimation of the molecular weight, SDS-PAGE [18] was conducted on a 5% stacking gel and a 12% separating gel. Gel filtration accompanied with high-performance liquid chromatography (HPLC) was performed to estimate the native molecular weight of the enzyme. The conditions were controlled as follows: Column: TSK G2000SWxl (Tosoh Bioscience LLC, Minato-ku, Tokyo, Japan); Mobile phase: 0.1 M phosphate buffer (pH 6.7) containing 0.05% (W/V) $NaN_3$ and 0.1 M $Na_2SO_4$; Flow rate: 1 ml $min^{-1}$; Detection: UV at 280 nm. The standard samples for gel filtration were thyroglobulin (bovine, MW: 670 kDa), γ-globulin (bovine, MW: 158 kDa), ovalbumin (chicken, MW: 44 kDa), myoglobin (horse, MW: 17 kDa), and vitamin B12 (MW: 1.35 kDa).

### 2.5. Assay of enzyme activity

Enzymatic reactions were performed in a 1 ml reaction system consisting of 1% inulin (W/V), 50 mM acetate buffer (pH 5.5), and 10 nM enzyme at 40 °C for 10 min, which was terminated by boiling. For the HPLC analysis of the reaction mixture, the supernatant of the mixture was filtered through a 0.22 μm membrane filter into a sample HPLC vial after centrifugation for 30 min at 20,000 × *g*. The HPLC system was equipped with a refractive index detector (Waters Alliance 2695, Waters Corporation, Milford, MA, USA) and a 4.6 mm × 250 mm Waters SugarPak1 column (Waters Corporation, Milford, MA, USA). The column temperature was set at 85 °C, and water was used as the mobile phase at a flow rate of 0.4 ml $min^{-1}$.