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Inulin fructotransferase (DFA III-producing) from *Leifsonia* sp. T88-4

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Abstract

An inulin fructotransferase (DFA III-producing) [EC 2.4.1.93] from *Leifsonia* sp. T88-4 was purified and characterized, for the first time. The enzyme was purified from culture supernatant of the microorganism 32.4-fold with a yield of 11.3% using a DEAE–Toyopearl chromatography and two times of Super Q-Toyopearl chromatography. The enzyme showed maximum activity at pH 5.0 and 65 °C. This maximum temperature was highest in the inulin fructotransferase (DFA III-producing) reported to date. The enzyme activity was stable up to 60 °C after 30 min heat treatment. The molecular mass of the enzyme was estimated to be 44 kDa by SDS–PAGE and 74 kDa by gel filtration, and was considered to be a dimer. The N-terminal amino acid sequence (16 amino acid residues) was determined as METNVYDVTDVGVPGR. The K_m value of the reaction at pH 5.0 and 60 °C was estimated to be 1.0 mM.

Keywords: *Leifsonia*; DFA III (difructose dianhydride III); Inulin

1. Introduction

Inulin is a storage polysaccharide contained in chicory, dahlia, Jerusalem artichoke, and other plants. The chemical structure of inulin is a β-2,1-linked fructose polymer terminated with a sucrose residue. In studies of inulin decomposing enzymes, inulinase [EC 3.2.1.7] from *Penicillum* sp. was reported (Nakamura & Hoashi, 1969). Afterwards, a new type of inulin decomposing enzyme produced by *Arthrobacter ureafaciens* was discovered (Uchiyama, Niwa, & Tanaka, 1973). The enzyme converted inulin into an oligosaccharide DFA III (di-D-fructofuranose 1,2′:2,3′ dianhydride) and a small amount of other oligosaccharides. This enzyme was designated as inulin fructotransferase (DFA III-producing) [EC 2.4.1.93]. Subsequently, there have been several reports on the inulin fructotransferase (DFA III-producing) from *Arthrobacter* species (Haraguchi et al., 1988; Haraguchi, Yamanaka, & Ohtsubo, 2002; Haraguchi, Yoshida, & Ohtsubo, 2005; Kawamura, Takahashi, & Uchiyama, 1988; Yokota, Enomoto, & Tomita, 1991). Kang, Kim, Chang, and Kim (1998) reported on the enzyme from *Bacillus* sp.

The DFA III has half the sweetness of sucrose. Recently, it was found that the DFA III accelerates the assimilation of minerals (Ca, Fe, and so on) from intestines (Saito & Tomita, 2000). Therefore, the DFA III has a potential for the improvement of osteoporosis and iron deficiency anemia. The sales of DFA III (brand name; Twintose) started in 2004 in Japan.

Recently, we isolated a microorganism, strain T88-4, which produced an inulin fructotransferase (DFA III-producing) in the culture supernatant. Through taxonomical studies, the microorganism was identified as *Leifsonia* sp. T88-4. In this paper, we describe the purification and properties of the inulin fructotransferase (DFA III-producing) from *Leifsonia* sp., for the first time.

2. Materials and methods

2.1. Identification of microorganism

We isolated a microorganism, strain T88-4, from a soil sample collected in Ibaraki prefecture, Japan (SIID3091,

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NCIMB Japan). For the extraction of a genome DNA, Prepman method (Applied Biosystems, Co. Ltd, USA) was used. The amplification of 16S rDNA was performed using PCR system 9600 (Applied Biosystems, Co. Ltd). The DNA sequencing was performed with an ABI PRISM 3100 sequencer (Applied Biosystems Co. Ltd)

2.2. Cultivation of microorganism

For a pre-culture, the microorganism was cultured in a 500 ml shaking flask at 30 °C, for 24 h which containing a medium (100 ml). The medium composed of 0.4% $Na_2HPO_4{\cdot}12H_2O$, 0.1% KH_2PO_4, 0.1% $NaNO_3$, 0.05% $MgSO_4{\cdot}7H_2O$, 0.001% $CaCl_2{\cdot}2H_2O$, 0.001% $FeSO_4{\cdot}7H_2O$, 0.05% yeast extract (Difco), and 0.3% inulin, pH 7.0. The pre-culture was inoculated in a 5 L Erlenmeyer flask containing 1 L of the same medium and cultured at 30 °C, for 24 h. After the cultivation, the cells were removed by centrifugation (8000*g*, 30 min) and the supernatant was used as a crude enzyme solution.

2.3. Standard assay methods

For the measurement of the enzyme activity, 0.1 M citrate buffer, pH 5.0 (0.5 ml), the enzyme solution (0.1 ml), water (0.4 ml), and 2% inulin (1.0 ml) were mixed. The enzyme reaction was performed at 60 °C, for 30 min, and the reaction was stopped by heating at 100 °C, for 7 min. The DFA III produced was determined by HPLC (column, Shim-pack CLC ODS, 4.6 mm × 25 cm (Shimadzu Co. Ltd, Kyoto); mobile phase, water; detector, refractive index detector). One unit of the enzyme was defined as the amount of enzyme which can produce 1 μmol of DFA III per min at pH 5.0 and 60 °C. Protein concentrations were determined by the method of Lowry, Rosebrough, Farr, and Randall (1951) using bovine serum albumin as a standard.

2.4. Purification of enzyme

The crude enzyme solution was dialyzed against, 10 mM Tris–HCl buffer, pH 8.5. The dialyzed enzyme solution was applied on a column of DEAE–Toyopearl 650 M (2.5 cm × 16 cm, Tohsoh Co. Ltd, Japan) equilibrated with 10 mM Tris–HCl buffer, pH 8.5. The elution was performed with linear 0–0.5 M NaCl gradient in the same buffer. The fractions showing the enzyme activity were pooled and dialyzed against 10 mM Tris–HCl buffer, pH 8.5. The dialyzed enzyme solution was applied on a column of Super Q-Toyopearl 650 M (1st, 1.5 cm × 12 cm) equilibrated with 10 mM Tris–HCl buffer, pH 8.5. The elution was performed with linear 0–0.5 M NaCl gradient in the same buffer. The fractions showing the enzyme activity were pooled and dialyzed against 5 mM sodium phosphate buffer, pH 8.0. The dialyzed enzyme solution was applied on a column of Super Q-Toyopearl 650M (2nd, 1.5 cm × 12 cm) equilibrated with 5 mM sodium phosphate buffer, pH 8.0. The elution was performed with linear 0–0.4 M NaCl gradient in the same buffer. The fractions showing the enzyme activity were pooled and used as a purified enzyme solution.

2.5. Estimation of molecular mass

The molecular mass of the enzyme was estimated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) using a ready-made gel (PAGEL: NPU-10 L, Atto Co. Ltd, Japan). Also, the molecular mass of the enzyme was estimated by gel filtration on HPLC (column, TSK-gel G3000SWXL, Tohsoh Co. Ltd, Japan; mobile phase, 100 mM sodium phosphate buffer, pH 7.0, containing 0.5 M NaCl; detection, UV 280 nm).

2.6. Amino acid sequencing

The purified enzyme was electrically blotted on a PVDF membrane (Sequi-Blot, Bio-Rad Co. Ltd, USA). And the amino acid sequence of N-terminal region of the enzyme was analyzed by automated Edman degradation with G1005A protein sequencer (Hewlett Packard Co. Ltd, USA).

2.7. Preparation of reaction products

For the preparation of the reaction products, 0.1 M citrate buffer, pH 5.5 (0.5 ml), the purified enzyme solution (2 ml, 7 U/ml), and 5% inulin (10 ml) were mixed. The enzyme reaction was performed at 60 °C, for 16 h and reaction was stopped by heating at 100 °C for 7 min. After cooling, the reaction mixture was analyzed by a paper chromatography. The paper chromatography was performed at 37 °C using Toyo No. 50 filter paper (Advantec Toyo, Co. Ltd, Japan) with a solvent system of *n*-butyl alcohol/pyridine/water (3:2:2, by volume). The chromatogram was irrigated twice. The spots of the reaction products were revealed with resorcinol–HCl reagent. An ion-exchange resin, Amberlite MB-3 (2.0 g, Organo Co. Ltd, Japan), was added to the rest of the reaction mixture and the suspension was kept 3 h at room temperature. After removing the resin by filtration by a filter paper,

Table 1
The taxonomic characteristics of strain T88-4

Shape and size	Rod; 0.6–0.7 × 2.0–3.0 μm
Gram staining	Positive
Spore formation	−
Motility	+
Catalase	+
Oxidase	−
Alkaline phosphatase	+
β-Galactosidase	+
α-Glucosidase	−
Reduction of nitrate	−
Urease	−
Gelatin hydrolysis	−

Table 2
Purification of inulin fructotransferase (DFA III-producing) from *Leifsonia* sp. T88-4

Step	Total protein (mg)	Total activity (units)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Crude enzyme	103	2054	19.9	1	100
DEAE–Toyopearl	6.38	814	128	6.43	39.6
Super Q-Toyopearl (1st)	1.07	420	393	19.7	20.4
Super Q-Toyopearl (2nd)	0.36	232	644	32.4	11.3

the reaction mixture was concentrated by freeze-drying. To separate the main reaction product, the freeze-dried sample was dissolved in 1 ml of water, and was applied on a column of Toyopearl HW40S (2.5 cm × 24 cm, Tosoh Co. Ltd, Japan) equilibrated with water. The elution was performed with water and the fractions containing the pure main product were pooled (The purity was checked by HPLC). The fraction was concentrated by freeze-drying and used as a sample of ^{13}C NMR. The ^{13}C NMR spectrum was recorded in D_2O with a Bruker Avance 500 spectrometer (Bruker Co. Ltd, Germany) using 3-trimethylsilyl-1-propanesulfonic acid sodium salt (DSS) as a standard material.

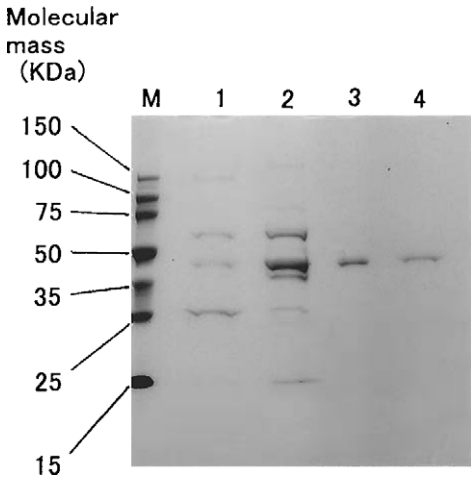

Fig. 1. SDS–PAGE of the intermediate fractions and the purified enzyme. Lane M, molecular mass standard markers; lane 1, crude enzyme; lane 2, DEAE–Toyopearl fraction; lane 3, Super Q-Toyopearl (first) fraction; lane 4, Super Q-Toyopearl (second) fraction (purified enzyme).

3. Results and discussion

3.1. Identification of microorganism

Table 1 summarizes the taxonomic characters of strain T88-4. The microorganism was a Gram-positive non-spore motile rod. It was catalase positive and oxidase negative. Therefore, strain T88-4 was estimated to be a coryneform bacterium. The 16S rDNA sequence showed homology of 99.2% with that of *Leifsonia poae* (type strain) and 98.5% with that of *Leifsonia xyli*. And on the molecular genealogical analysis of the 16S rDNA sequence, the strain T88-4 formed a same cluster with that of *L. poae* (data not shown). From these results, the strain T88-4 was identified as *Leifsonia* sp. T88-4.

3.2. Purification of enzyme

Table 2 shows a summary of the enzyme purification. The enzyme was purified 32.4-fold with a yield of 11.3% by a DEAE–Toyopearl chromatography and two times of Super Q-Toyopearl chromatography. The purified enzyme was analyzed by SDS–PAGE, and as shown in Fig. 1, it gave a single band.

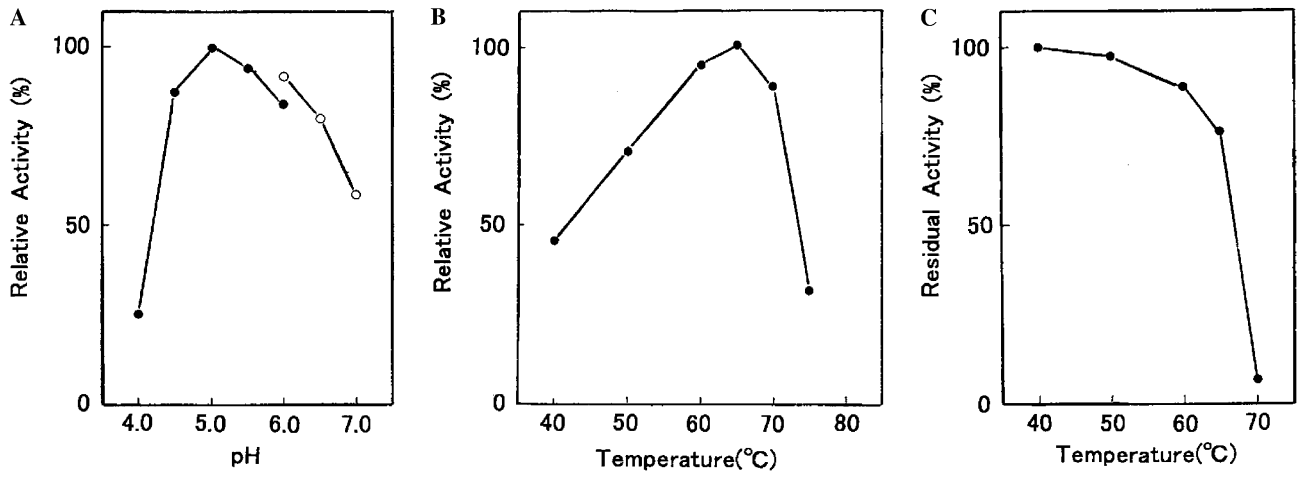

Fig. 2. (A) Effect of pH on the enzyme activity. (●), citrate buffer; (○), phosphate buffer. (B) Effect of temperature on the enzyme activity. (C) Thermal stability of the enzyme.

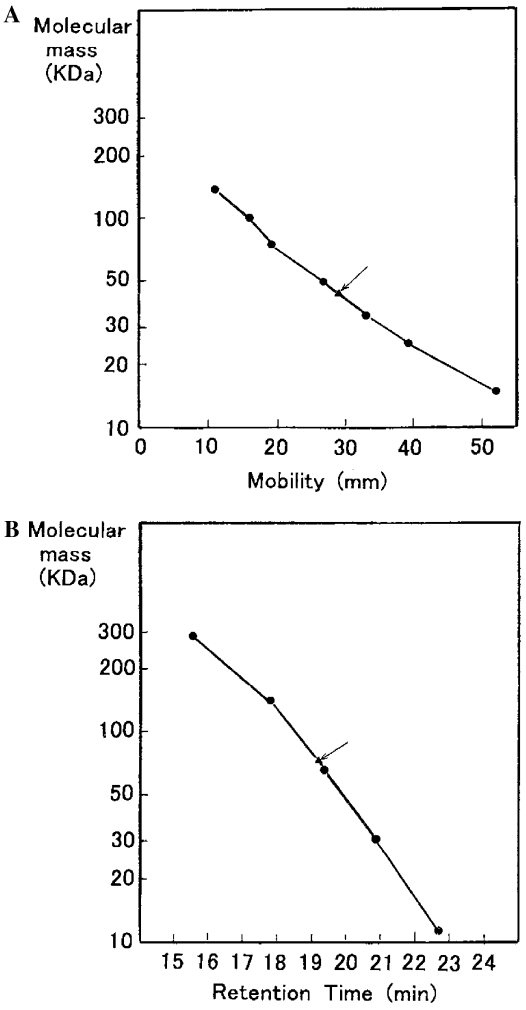

Fig. 3. (A) Estimation of the molecular mass by SDS–PAGE. Standard marker proteins, Takara perfect protein markers (150, 100, 75, 50, 35, 25, 15 kDa). (B) Estimation of the molecular mass by gel filtration. Standard marker proteins, glutamate dehydrogenase (290 kDa); lactate dehydrogenase (142 kDa); enolase (67 kDa); myokinase (32 kDa); cytochrome *c* (12.4 kDa).

3.3. Effect of pH and temperature on enzyme activity

The effect of pH on the enzyme activity was investigated in the pH range 4.0–7.0 at 60 °C. As shown in Fig. 2(A), maximum activity was obtained at pH 5.0. This optimum pH was lowest in the inulin fructotransferases (DFA III-producing) (same as that of *A. globiformis* C11-1 (Haraguchi et al., 1988)). The enzyme reaction was performed in the range 30–75 °C at pH 5.0. As shown in Fig. 2(B), maximum activity was obtained at 65 °C. This maximum temperature was highest in the inulin fructotransferase (DFA III-producing) reported to date. For the industrial application of the enzyme, the high reaction temperature is important because of the sanitation. Therefore, it will be advantageous to use this enzyme for the production of DFA III.

3.4. Thermal stability

The enzyme solution was heated at various temperatures for 30 min at pH 5.0, after that the residual activities were measured at pH 5.0 and 60 °C. As shown in Fig. 2(C), the enzyme was stable up to 60 °C, but it was inactivated at 70 °C.

3.5. Molecular mass estimation

Fig. 3(A) shows plots of logarithmic molecular mass of the enzyme vs. protein mobility on SDS–PAGE. The molecular mass of the enzyme was estimated to be 44 kDa. Fig. 3(B) shows the result of molecular mass estimation by gel filtration with TSK-gel G3000SWXL. The molecular mass was estimated to be 74 kDa. From these results, the enzyme was considered to be a dimer. Table 3 summarizes the comparison of some properties of inulin fructotransferases (DFA III-producing) from different microorganisms. In the inulin fructotransferase (DFA III-

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C11-1 ADGQQGAPLNSPNTYDVT
H65-7 ADSTEETN-----RYDVT
Snu-7 ADGQDGAPLNQVNTYD--
T13-2 AQDAKAGPFNSPNTYDVT
T88-4 ME----TN-----VYDVT
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Fig. 4. Comparison of N-terminal amino acid sequence of inulin fructotransferase (DFA III-producing) from various microorganisms. C11-1, the sequence of *A. globiformis* C11-1; H65-7, the sequence of *Arthrobacter* sp. H65-7; Snu-7, the sequence of *Bacillus* sp. Snu-7; T13-2, the sequence of *A. pascens* T13-2; T88-4, the sequence of *Leifsonia* sp. T88-4. The identical residues are presented by white letters in black boxes.

Table 3
The comparison of properties of inulin fructotransferases (DFA III-producing) from different microorganisms

Microorganism	Optimum		Heat stability		Molecular mass (kDa)		K_m (mM)	References
	pH	°C	°C	Time (min)	SDS–PAGE	Gel filtration		
Leifsonia sp. T88-4	5.0	65	60	30	44	74	1.0	This work
A. ureafaciens	6.0	50	50	30		80		Uchiyama et al. (1973)
A. globiformis C11-1	5.0	55	75	20	45	50		Haraguchi et al. (1988)
A. ilicis OKU17B	5.5	60	70	30	27	50		Kawamura et al. (1988)
Arthrobacter sp. H65-7	5.5	60	70	20	49	100	0.8	Yokota and Hirayama et al. (1991)
A. pascens T13-2	5.5–6.0	50	75	20	44	79		Haraguchi et al. (2002)
Arthrobacter sp. L68-1	5.5–6.0	55	80	60	43	73	10	Haraguchi et al. (2005)
Bacillus sp. snu-7	6.0	40	60	10	62		5.4	Kang et al. (1998)

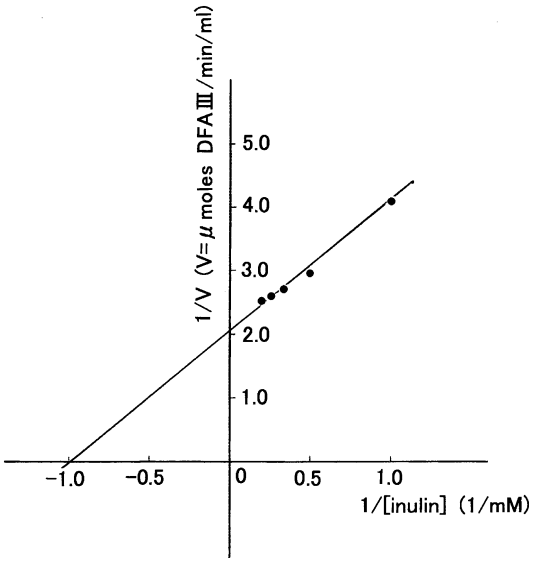

Fig. 5. Estimation of K_m value of the enzyme by double reciprocal plots.

producing), the enzymes from *Arthrobacters* were mainly reported. This is the first report of the enzyme from *Leifsonia* sp.

3.6. N-terminal amino acid sequence

N-terminal amino acid sequence was determined as METNVYDVTDVGVPGR. Fig. 4 shows the comparison of N-terminal amino acid sequence of inulin fructotransferase (DFA III-producing) from various microorganisms. It was reported that the enzyme from *Arthrobacter* sp. H65-7 (Sakurai, Yokota, & Tomita, 1997) and the enzyme from *A. globiformis* C11-1 (Haraguchi, Mori, & Hayashi, 2000), have a signal peptide for secretion. While the N-terminal amino acid residue of the enzyme from *Leifsonia* sp. T88-4 was M. This result implies that this enzyme does not have a signal peptide for secretion.

3.7. Estimation of K_m value

The enzyme reaction was performed at pH 5.0 and 60 °C at various concentrations of inulin (molecular mass assumed as 5000 Da). The double-reciprocal plots of the reaction rate against the substrate concentrations were performed. As shown in Fig. 5, the K_m value at the conditions was estimated to be 1.0 mM. This K_m value was near to that of *Arthrobacter* sp. H65-7 (Yokota & Enomoto et al., 1991).

Table 4
^{13}C NMR chemical shifts of main reaction product and DFA III

Assignment carbon atom number	Chemical shifts of ^{13}C NMR			
	Main reaction product		[a]Standard DFA III	
	α	β	α	β
1	66.1	61.7	66.2	61.7
2	106.3	104.1	106.3	104.2
3	83.9	84.5	83.9	84.5
4	78.0	74.9	78.1	75.0
5	82.8	81.7	82.9	81.8
6	63.3	63.8	63.3	63.8

[a] Reference: Yokota and Hirayama et al. (1991).

3.8. Reaction products

The reaction mixture, after the exhaustive enzyme reaction, was analyzed by paper chromatography as mentioned in Section 2. The R_f value for the main reaction product and two residual oligosaccharides (minor products) were 0.96, 0.47, and 0.38, respectively. The R_f values for the standard materials (DFA III, GF_2 (1-kestose), GF_3 (nystose), GF_4 (fructofuranosyl nystose)) were 0.96, 0.59, 0.48, and 0.39, respectively (data not shown). Therefore, the residual oligosaccharides (minor products) were estimated to be GF_3 and GF_4. The main reaction product was prepared from inulin by the method described in materials and methods.

The ^{13}C NMR spectrum of the main reaction product was recorded. As shown in Table 4, ^{13}C NMR data of the main reaction product agreed well with those of standard DFA III (Yokota & Hirayama et al., 1991).

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