

## 研究ノート

Purification and Properties of Inulin Fructotransferase (DFA -Producing)  
from *Arthrobacter* sp. B30-2.

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## Abstract

An inulin fructotransferase (DFA -producing) [EC 2.4.1.93] from *Arthrobacter* sp. B30-2 was purified and characterized. The enzyme was purified 7.8-fold from the culture supernatant of the microorganism with a yield of 23% by using DEAE-Toyopearl chromatography, butyl-Toyopearl chromatography, and Super-Q Toyopearl chromatography. The enzyme showed maximum activity at pH 6.0 and 55 °C. The enzyme activity was stable up to 80 °C for 30 min. The specific activity of the purified enzyme was 1190 units/mg protein. This specific activity is the highest reported to date. The molecular mass of the enzyme was estimated to be 44 kDa by SDS-PAGE and 70 kDa by gel filtration, and thus the enzyme was considered to be a dimer. The N-terminal amino acid sequence (14 amino acid residues) was determined as ADSTEETNRYDVTS.

Key words: *Arthrobacter*, DFA (difructose dianhydride), inulin.

## Introduction

Inulin is a polysaccharide found in chicory, dahlia, Jerusalem artichoke, and other plants. Inulin is a  $\beta$ -2,1 linked fructose polymer terminated by a sucrose residue. Past studies have reported inulin-decomposing enzymes, including inulinase [EC 3.2.1.7], from yeasts and molds. Later, a new type of inulin-decomposing enzyme produced by *Arthrobacter ureafaciens* was discovered<sup>1)</sup>. The enzyme converted inulin into the oligo-saccharide DFA (di-D-fructofuranose 1,2':2, 3' dianhydride) and a small amount of other oligo-saccharides. This enzyme was designated as inulin fructotransferase (DFA -producing) [EC 2.4.1.93]. Subsequently, there have been several reports of inulin fructotransferase (DFA -producing) from other *Arthro-*

*bacter* species<sup>2-6)</sup>. Kang et al.<sup>7)</sup> has reported the enzyme from *Bacillus* sp., while we reported the enzyme from *Leifsonia* sp.<sup>8)</sup>.

It was found that DFA accelerates the assimilation of minerals (Ca, Fe, and so on) in the intestines<sup>9)</sup>. Therefore, DFA has the potential for use in the treatment of osteoporosis and iron-deficient anemia. DFA has been for sale in Japan since 2004. Recently, we isolated a microorganism, strain B30-2, that produced an inulin fructotransferase (DFA -producing) in the culture supernatant. Through taxonomic studies, the microorganism was identified as *Arthrobacter* sp. B30-2. The enzyme produced by *Arthrobacter* sp. B30-2 strain has a high specific activity. In this paper we describe the purification and the characteristics of this enzyme.

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## Materials and Methods

### Chemicals

An analytical standard of DFA was obtained from FancI Co. Ltd., Japan. The standard fructo-oligosaccharides (1-kestose, GF<sub>2</sub>; nystose, GF<sub>3</sub>; and fructofuranosyl nystose, GF<sub>4</sub>) were obtained from Wako Pure Chemicals Co. Ltd., Japan.

### Cultivation of the microorganism

For pre-culture, the microorganism was cultured in a 500-ml shaking flask at 30 °C for 24 h. The medium (100 ml/flask) was composed of 0.4% Na<sub>2</sub>HPO<sub>4</sub>•12H<sub>2</sub>O, 0.1% KH<sub>2</sub>PO<sub>4</sub>, 0.1% NaNO<sub>3</sub>, 0.05% MgSO<sub>4</sub>•7H<sub>2</sub>O, 0.001% CaCl<sub>2</sub>•2H<sub>2</sub>O, 0.001% FeSO<sub>4</sub>•7H<sub>2</sub>O, 0.05% yeast extract (Difco), and 0.3% inulin, adjusted to pH 7.0. The pre-culture (100 ml) was inoculated in a 5-l Erlenmeyer flask containing 1 l of the same medium and cultured at 30 °C for 24 h. After cultivation, the cells were removed by centrifugation (8000 × *g* for 30 min) and the supernatant was used as a crude enzyme solution.

### Standard assay methods

For the measurement of enzyme activity, 0.1 M phosphate buffer, pH 6.0 (0.5 ml), enzyme solution (0.02 ml), water (0.48 ml), and 2% inulin (1.0 ml) were mixed. The reaction was performed at 55 °C for 30 min, and then stopped by heating to 100 °C for 7 min. The DFA 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 that can produce 1 μmol of DFA per min at pH 6.0 and 55 °C. Protein concentrations were determined by using the method of Lowry et al.<sup>10)</sup> using bovine serum albumin as a standard.

### Purification of the enzyme

The crude enzyme solution was dialyzed against 10 mM Tris-HCl buffer (pH 8.5). The dialyzed enzyme solution was applied to a column of DEAE-Toyopearl 650M (2.5 cm × 17 cm, Tohsoh Co. Ltd, Japan) equilibrated with 10 mM Tris-HCl buffer (pH 8.5). Elution was performed with a linear NaCl gradient of 0 to 0.5 M in the

same buffer. Fractions showing enzyme activity were pooled and dialyzed against 10 mM Tris-HCl buffer (pH 8.0) containing 120 g/l ammonium sulfate. The enzyme solution was applied to a column of butyl-Toyopearl 650 M (1.5 cm × 12 cm) equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing ammonium sulfate (120 g/l). Elution was performed with a linear ammonium sulfate gradient of 120 to 0 g/l in the same buffer. Fractions displaying enzyme activity were pooled and dialyzed against 5 mM phosphate buffer (pH 8.0). The dialyzed enzyme solution was applied to a column of Super-Q Toyopearl 650M (1.5 cm × 12 cm) equilibrated with 5 mM phosphate buffer (pH 8.0). The elution was performed with a linear NaCl gradient of 0 to 0.4 M in the same buffer. Fractions containing enzyme activity were pooled and used as a purified enzyme solution.

### Estimation of molecular mass

The molecular mass of the enzyme was estimated by using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a ready-made gel (PAGEL: NPU-10L, Atto Co. Ltd., Japan). Also, the molecular mass 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).

### Amino acid sequencing

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

### Preparation of reaction products

For the preparation of the reaction products, 0.1 M phosphate buffer, pH 6.0 (0.5 ml), the purified enzyme solution (0.5 ml, 18 units), and 5% inulin (2 ml) were mixed. The enzyme reaction was performed at 55 °C for 17 h and stopped by heating to 100 °C for 7 min. After cooling, the reaction mixture was analyzed by paper chromatography. The paper chromatography was performed at 37 °C by using Toyo No. 50 filter paper (Advantec Toyo, Co. Ltd., Japan) with a solvent system of *n*-butyl alcohol:

pyridine: water (3: 2: 2, v/v). The chromatogram was irrigated twice. The spots of the reaction products were visible following resorcinol-HCl reagent treatment.

## Results and discussion

### Identification of the microorganism

Table 1 summarizes the taxonomic characteristics of the B30-2 strain. The microorganism was a Gram-positive non-spore forming bacterium. It was catalase positive and oxidase negative. Therefore, strain B30-2 was estimated to be a coryneform bacterium. The 16S rDNA sequence showed a 99.1% homology with that of *Arthrobacter oxidans* DSM20119 (type strain), although upon molecular genealogical analysis of the 16S rDNA sequence, the strain B30-2 did not agree with any species of *Arthrobacter* (data not shown). Therefore, the strain was designated as *Arthrobacter* sp. B30-2.

### Purification of the enzyme

The enzyme was purified 7.8-fold with a yield of 23% by using DEAE-Toyopearl chromatography, butyl-Toyopearl chromatography and Super-Q Toyopearl chromatography. During the purification procedure, the fractions were analyzed by SDS-PAGE (Fig. 1). The Super-Q Toyopearl fraction gave a single band. A summary of the purification is presented in Table 2. The specific activity of the purified enzyme from *Arthrobacter* sp. B30-2 was 1190 units/mg protein. Table 3 shows a comparison of the specific activity of inulin fructotransferases (DFA - producing) from different microorganisms. It is notable that the specific activity of the enzyme of *Arthrobacter* sp. B30-2 was the highest reported to date.

### Effect of pH and temperature on enzyme activity

The effect of pH on enzyme activity was investigated in the pH range of 4.0 to 7.5 at 55 °C. As shown in Fig. 2 (A), maximum activity was obtained at pH 6.0. The en-

zyme reaction was performed over a temperature range of 30 to 75 °C at pH 6.0, and the maximum activity was obtained at 55 °C (Fig. 2(B)).

### Thermal stability

The enzyme solution was heated to various temperatures for 30 min at pH 6.0, after which the residual activities were measured at pH 6.0 and 55 °C. As shown in Fig.

Table 1. The taxonomic characteristics of strain B30-2

Shape and size	Rod; 0.65 x 2.5 µm
Gram staining	Positive
Spore formation	-
Motility	-
Pleomorphism	+
Catalase	+
Oxidase	-
Reduction of nitrate	+
Gelatin hydrolysis	-

### Molecular mass (KDa)

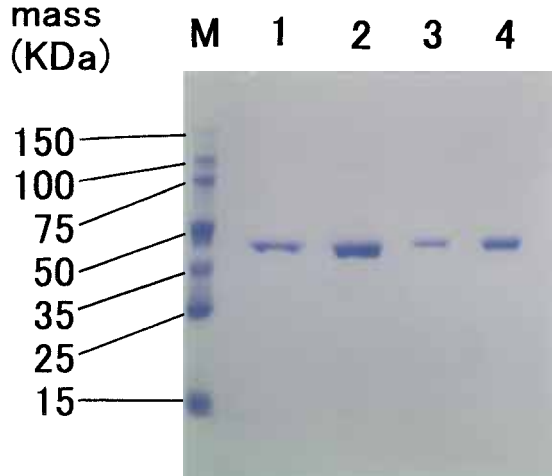


Fig. 1. SDS-PAGE of intermediate fractions and the purified enzyme.

Table 2. Purification of inulin fructotransferase (DFA -producing) from *Arthrobacter* sp. B30-2

Step	Total activity (units)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Crude enzyme	8990	58.7	153	1	100.0
DEAE-Toyopearl	2600	5.1	510	3.33	28.9
Butyl-Toyopearl	2370	4.41	537	3.51	26.4
SuperQ-Toyopearl	2030	1.71	1190	7.78	22.6

Table 3. The comparison of specific activity of inulin fructotransferases(DFA -producing) from different microorganisms

Microorganism	Specific activity (units/ mg protein)	References
<i>Arthrobacter</i> sp. B30-2	1190	This work
<i>Arthrobacter</i> sp. L68-1	933	6)
<i>Arthrobacter ilicis</i> OKU17B	853	3)
<i>Leifsonia</i> sp. T88-4	644	8)
<i>Arthrobacter</i> sp. H65-7	604	4)
<i>Arthrobacter globiformis</i> C11-1	294	2)
<i>Bacillus</i> sp. snu-7	45.8	7)

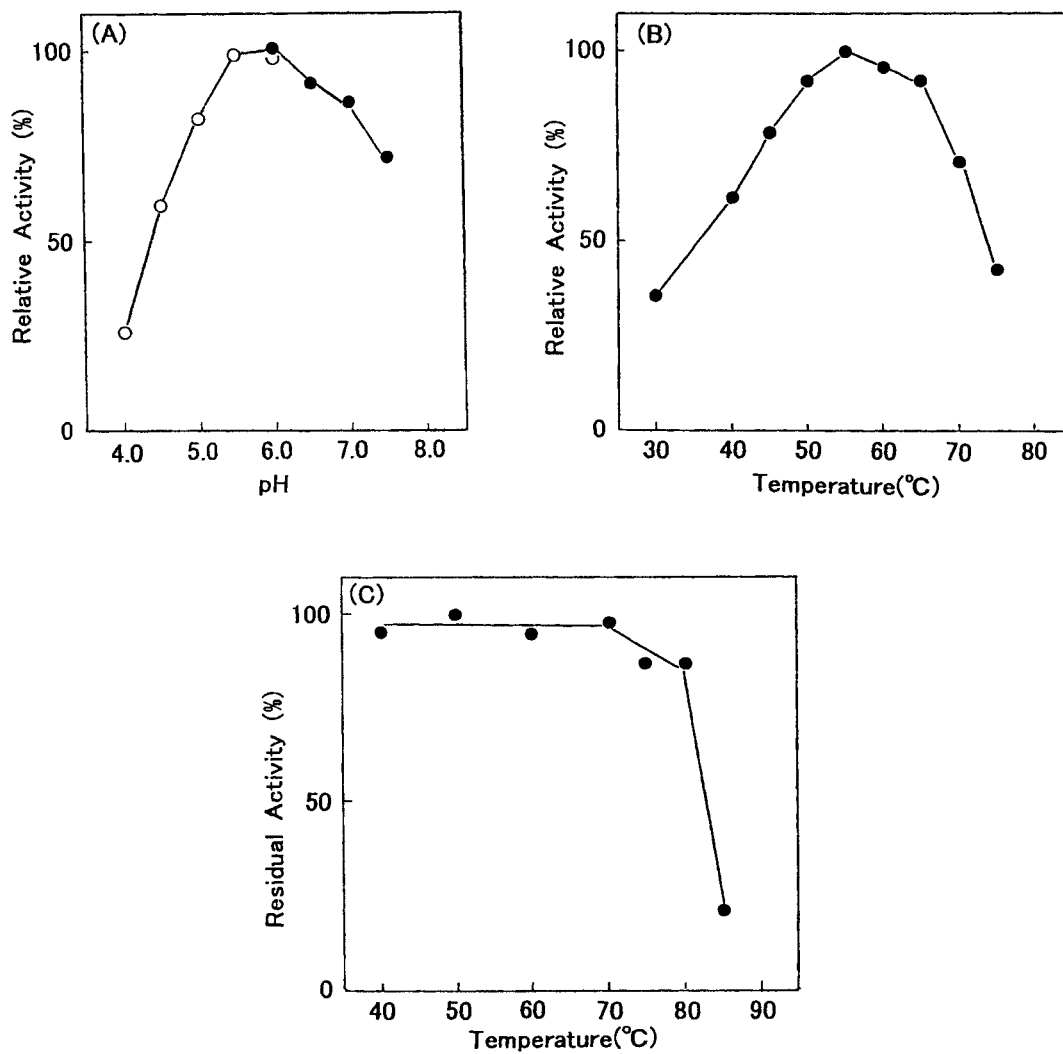


Fig. 2.

(A) The effect of pH on enzyme activity. (○), citrate buffer; (●), phosphate buffer.

(B) The effect of temperature on enzyme activity.

(C) Thermal stability of the enzyme.

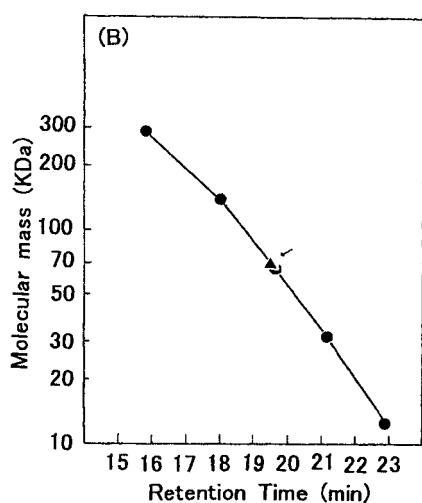
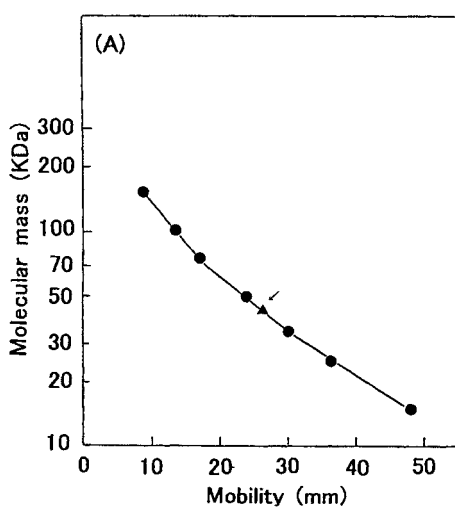


Fig. 3.

(A) The estimation of molecular mass by SDS-PAGE. Standard marker proteins, Takara perfect protein markers (150; 100; 75; 50; 35; 25; 15 kDa)

(B) The estimation of 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). The arrows show sample data.



Fig. 4.

The comparison of the *N*-terminal amino acid sequences of inulin fructotransferase (DFA -producing) from different microorganisms. B30-2, *Arthrobacter* sp. B30-2; C11-1, *A. globiformis* C11-1; H65-7, *Arthrobacter* sp. H65-7; Snu-7; *Bacillus* sp. Snu-7; T13-2, *A. pascens* T13-2. Identical residues are presented by white letters in black boxes.

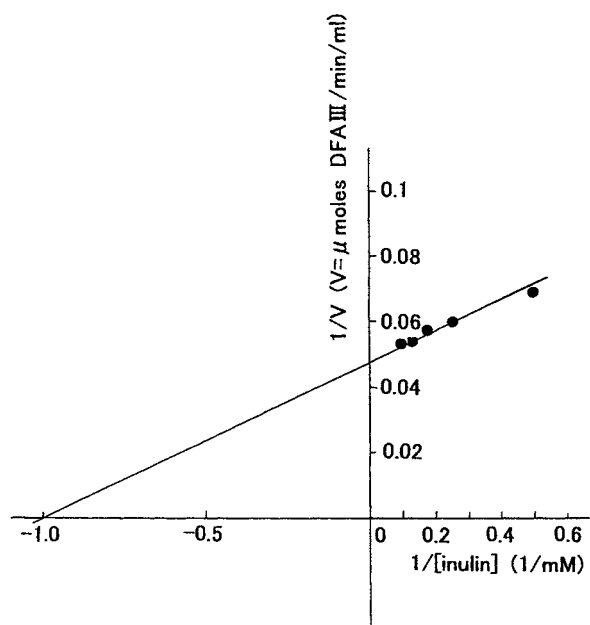
Fig. 5. Estimation of  $K_m$  value by double reciprocal plots

Table 4. The comparison of properties inulin fructotransferase (DFA -producing) from different microorganisms

Microorganism	Optimum pH	Optimum temp. (°C)	Heat stability (°C)	molecular mass(kDa)		References
				SDS-PAGE	Gel-Filtration	
<i>Arthrobacter</i> sp. B30-2	6	55	80	44	70	This work
<i>A. ureafaciens</i>	6	50	50		80	1)
<i>A. globiformis</i> C11-1	5	55	75	45	50	2)
<i>A. ilicis</i> OKU17B	5.5	60	70	27	50	3)
<i>Arthrobacter</i> sp. H65-7	5.5	60	70	49	100	4)
<i>A. pascens</i> T13-2	5.5-6.0	50	75	44	79	5)
<i>Arthrobacter</i> sp. L68-1	5.5-6.0	55	80	43	73	6)
<i>Bacillus</i> sp. snu-7	6	40	60	62		7)
<i>Leifsonia</i> sp. T88-4	5	65	60	44	74	8)

2 (C), the enzyme was stable up to 80 °C, but was inactivated above 85 °C. For industrial applications of the enzyme, heat stability is an important factor, and thus the enzyme is amenable to the large scale production of DFA.

#### Molecular mass estimations

Figure 3 (A) shows the logarithmic plots of enzyme molecular mass versus protein mobility on SDS-PAGE. The molecular mass of the enzyme was estimated to be 44 kDa. However, the molecular mass was estimated by gel filtration with TSK-gel G3000SWXL as 70 kDa. From these results, the enzyme is hypothesized to be a dimer. A comparison of some properties of inulin fructotransferases (DFA-producing) from different microorganisms is shown in Table 4.

#### N-terminal amino acid sequence

The N-terminal amino acid sequence was determined to be ADSTEETNRYDVTS. A comparison of N-terminal amino acid sequences of inulin fructotransferases (DFA-producing) from various microorganisms is summarized in Fig. 4. At the 14 N-terminal amino acid residues, the sequence of B30-2 was same as that of *Arthrobacter* sp. H65-7, yet the properties of the enzyme from *Arthrobacter* sp. B30-2 were different from those from *Arthrobacter* sp. H65-7 (heat stability, molecular mass, and so on; Table 4).

#### Estimation of Km value

The enzyme reaction was performed at pH 6.0 and 55 °C at various concentrations of inulin (molecular mass assumed to be 5,000 Da). Double-reciprocal plots of the reaction rate against the substrate concentrations were constructed and, as shown in Fig. 5, the Km value under the conditions was estimated to be 1 mM.

#### Reaction products

The reaction mixture produced following a lengthy reaction was analyzed by paper chromatography as described. The Rf values for the main reaction product and two residual oligo-saccharides (minor products) were 0.99, 0.48, and 0.39, respectively. The Rf values for the standard materials—DFA, GF<sub>2</sub> (1-kestose), GF<sub>3</sub> (nystose), and GF<sub>4</sub> (fructofuranosyl nystose)—were 0.98, 0.55, 0.47,

and 0.38, respectively (data not shown). Therefore, the residual oligo-saccharides (minor products) were hypothesized to be GF<sub>3</sub> and GF<sub>4</sub>.

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## ***Arthrobacter* sp. B30-2の Inulin fructotransferase (DFA -producing) の精製と性質**

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*Arthrobacter* sp. B30-2が生産する DFA 合成酵素を精製し、その性質を解明した。精製された本酵素の比活性は1190 unit/ mg protein に達し、これまでに報告された DFA 合成酵素のなかで最高であった。本酵素の反応至適 pH は6.0, 反応至適温度は55 °C であっ

た。本酵素は30分間の加熱に対して80 °C まで安定であった。本酵素の分子量については SDS-PAGE から44 kDa, ゲル濾過から70 kDa という値が得られ、2量体酵素と推察された。