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

研究ノート

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

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Abstract

An inulin fructotransferase (DFA Ⅲ-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 ℃. The enzyme activity was stable up to 80 ℃ 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 Ⅲ(difructose dianhydride Ⅲ), inulin.

Introduction

Inulin is a polysaccharide found in chicory, dahlia, Jerusalem artichoke, and other plants. Inulin is a β-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[1]. The enzyme converted inulin into the oligo-saccharide DFA Ⅲ(di-D-fructofuranose 1,2':2, 3' dianhydride) and a small amount of other oligo-saccharides. This enzyme was designated as inulin fructotransferase (DFA Ⅲ-producing) [EC 2.4.1.93]. Subsequently, there have been several reports of inulin fructotransferase (DFA Ⅲ-producing) from other *Arthrobacter* species[2-6]. Kang et al.[7] has reported the enzyme from *Bacillus* sp., while we reported the enzyme from *Leifsonia* sp[8].

It was found that DFA Ⅲ accelerates the assimilation of minerals (Ca, Fe, and so on) in the intestines[9]. Therefore, DFA Ⅲ has the potential for use in the treatment of osteoporosis and iron-deficient anemia. DFA Ⅲ has been for sale in Japan since 2004. Recently, we isolated a microorganism, strain B30-2, that produced an inulin fructotransferase (DFA Ⅲ-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 Ⅲ was obtained from Fancl Co. Ltd., Japan. The standard fructo-oligosaccharides (1-kestose, GF_2; nystose, GF_3; and fructofuranosyl nystose, GF_4) 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 ℃ for 24 h. The medium (100 ml/flask) was composed of 0.4% Na_2HPO_4•$12H_2O$, 0.1% KH_2PO_4, 0.1% $NaNO_3$, 0.05% $MgSO_4$•$7H_2O$, 0.001% $CaCl_2$•$2H_2O$, 0.001% $FeSO_4$•$7H_2O$, 0.05% yeast extract (Difco), and 0.3% inulin, adjusted to pH 7.0. The preculture (100 ml) was inoculated in a 5-l Erlenmeyer flask containing 1 l of the same medium and cultured at 30 ℃ 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 ℃ for 30 min, and then stopped by heating to 100 ℃ 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 that can produce 1 μmol of DFA Ⅲ per min at pH 6.0 and 55 ℃. Protein concentrations were determined by using the method of Lowry et al.[10] 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 ℃ for 17 h and stopped by heating to 100 ℃ for 7 min. After cooling, the reaction mixture was analyzed by paper chromatography. The paper chromatography was performed at 37 ℃ 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 III-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 ℃. As shown in Fig. 2 (A), maximum activity was obtained at pH 6.0. The enzyme reaction was performed over a temperature range of 30 to 75 ℃ at pH 6.0, and the maximum activity was obtained at 55 ℃ (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 ℃. As shown in Fig.

Table 1. The taxonomic characteristics ofstrain 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	−

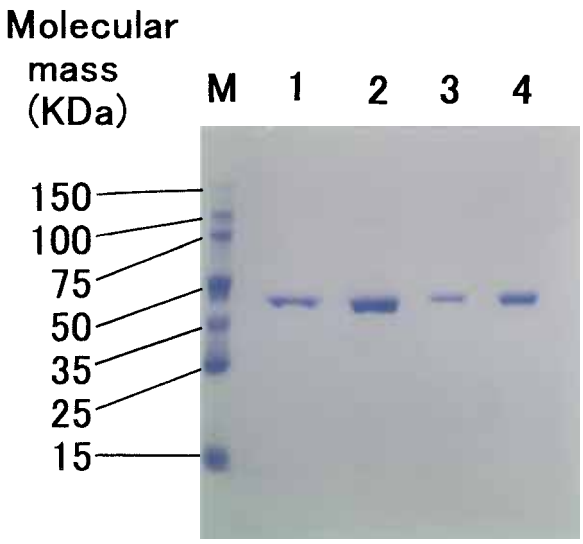

Fig. 1.
SDS-PAGE of intermediate fractions and the purified enzyme.

Table 2. Purification of inulin fructotransferase (DFA III-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 III-producing) from different microorganisms

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

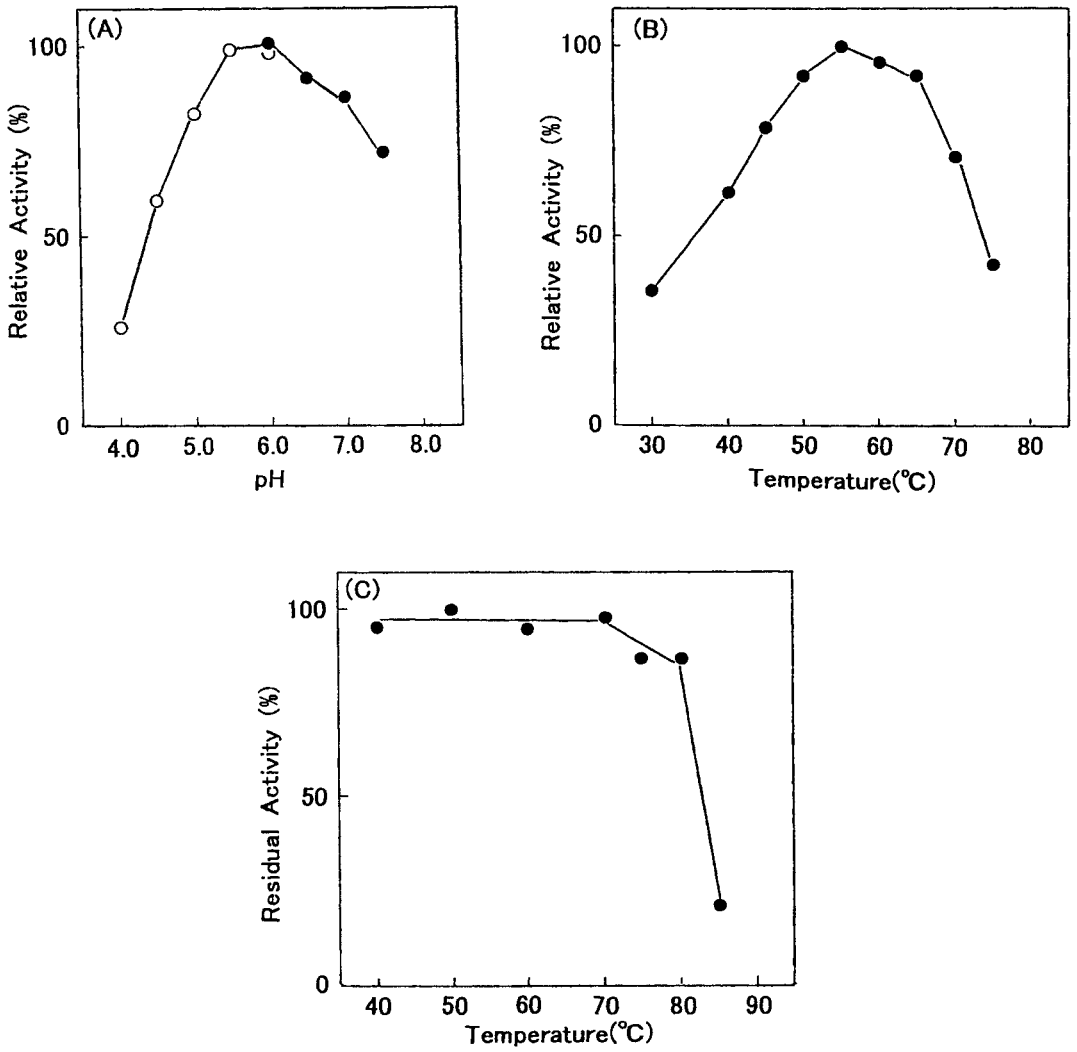

Fig. 2.

(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.

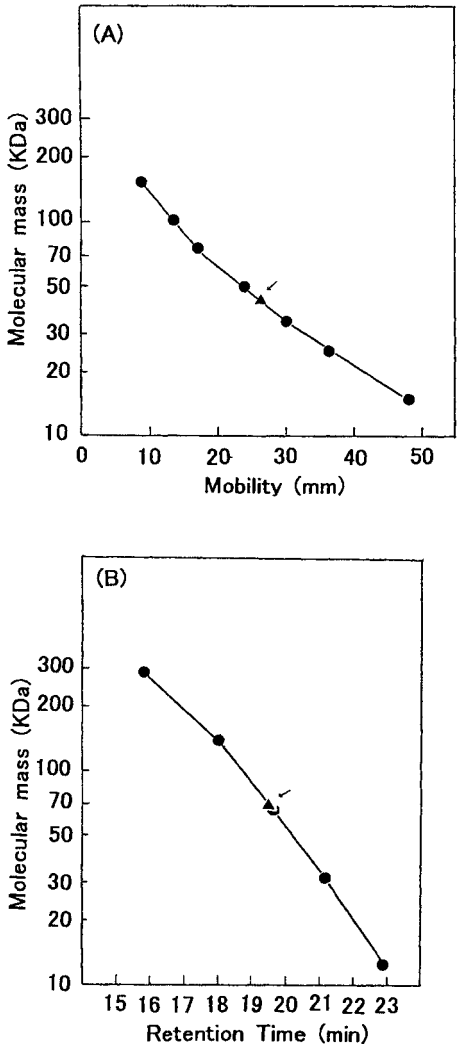

Fig. 3.

(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); cytchrome C (12.4 kDa). The arrows show sample data.

Fig. 4.

The comparison of the *N*-terminal amino acid sequences of inulin fructotransferase (DFA Ⅲ-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.

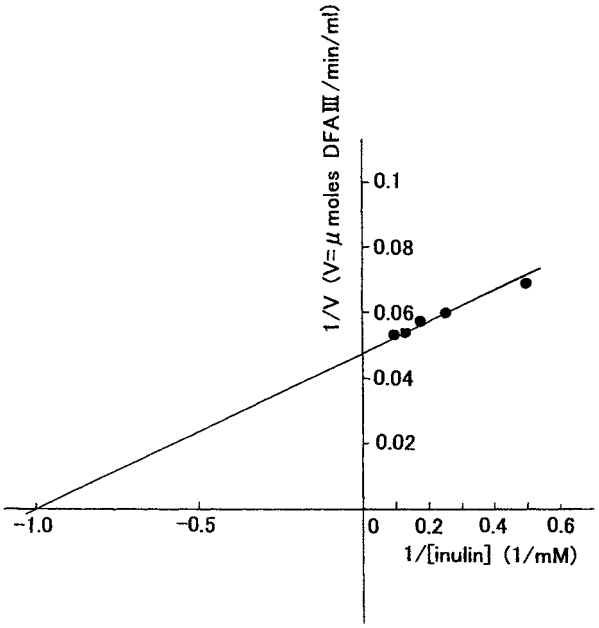

Fig. 5. Estimation of Km value by double reciprocal plots

Table 4. The comparison ofpropertiesinulin fructotransferase (DFA III-producing) from different microorganisms

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

2 (C), the enzyme was stable up to 80 ℃, but was inactivated above 85 ℃. 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 Ⅲ.

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 Ⅲ-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 Ⅲ-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 ℃ 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 Ⅲ, GF_2 (1-kestose), GF_3 (nystose), and GF_4 (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_3 and GF_4.

References

1) Uchiyama, T., Niwa, S., & Tanaka, K. Purification and properties of *Arthrobacter ureafaciens* inulase Ⅱ. *Biochim. Biophys. Acta*, 315, 412-420 (1973).

2) Haraguchi, K., Kishimoto, M., Seki, K., Kobayashi, S., & Kainuma, K. Purification and properties of inulin fructotransferase (depolymerizing) from *Arthrobacter globiformis* C11-1. *Agric. Biol. Chem.*, 52, 291-292 (1988).

3) Kawamura, M., Takahashi, S., & Uchiyama, T. Purification and some properties of inulin fructotransferase (depolymerizing) from *Arthrobacter ilicis. Agric. Biol. Chem.*, 52, 3209-3210 (1988).

4) Yokota, A., Enomoto, K., & Tomita, F. Purification and properties of an inulin fructotransferase (depolymerizing) from *Arthrobacter* sp. H65-7. *J.Ferment. Bioeng.*, 72, 262-265 (1991).

5) Haraguchi, K., Yamanaka, T., & Ohtsubo, K. Purification and properties of a heat stable inulin fructotransferase (DFA Ⅲ-producing) from *Arthrobacter pascens* T13-2. *Carbohydr. Polym.*, 52, 117-121 (2002).

6) Haraguchi, K., Yoshida, M. & Ohtsubo, K. Thermostable inulin fructotransferase (DFA Ⅲ-producing) from *Arthrobacter* sp. L68-1. *Carbohydr. Polym.*, 59, 411-416 (2005).

7) Kang, S., Kim, W., Chang, Y., & Kim, S. Purification and properties of inulin fructotransferase (DFA Ⅲ-producing) from *Bacillus* sp. snu-7. *Biosci. Biotec. Biochem.*, 62, 628-631 (1998).

8) Haraguchi, K., Yoshida, M., & Ohtsubo K. Inulin fructotransferase (DFA Ⅲ-producing) from *Leifsonia* sp. T 88-4., *Carbohydr. Polym.*, 66, 75-80 (2006).

9) Saito, K., & Tomita, F. Difructose anhydrides: Their mass production and physiological functions, *Biosci. Biotech. Biochem.*, 64, 1321-1327 (2000).

10) Lowry, O. H., Rosebrough, N. J., Farr A.L., & Randall R.J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, 193, 265-275 (1951).

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

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Arthrobacter sp B30-2が生産する DFA Ⅲ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 Ⅲ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℃であった．本酵素は30分間の加熱に対して80℃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．