# Arthrobactersp. B69-5のInulin fructotransferase (DFA I-producing)の精製と性質

研究ノート

# Purification and Properties of an Inulin Fructotransferase (DFA Ⅰ-Producing) from *Arthrobacter* sp. B69-5.

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## Abstract

An inulin fructotransferase (DFA I-producing) [EC 2.4.1.200] from *Arthrobacter* sp.B69-5 was purified and the characters of the enzyme were investigated. The enzyme was purified from a culture supernatant of the microorganism by 9.41 fold with a yield of 17.9 % using a DEAE- Toyopearl chromatography and a Butyl-Toyopearl chromatography. The enzyme showed maximum activity at pH 5.5 and 45 ℃. The enzyme activity was stable up to 75 ℃. The molecular mass of the enzyme was estimated to be 40 kDa by SDS-PAGE and 42 kDa by gel filtration, and was considered to be a monomer. The N-terminal amino acid sequence (20 amino acid residues) was determined as ANTVYDVTTWSGATISPYVD.

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

## Introduction

Inulin is a polysaccharide contained in chicory, dahlia, Jerusalem artichoke and other plants. The chemical structure of inulin is a beta-2, 1 linked fructose polymer terminated with a sucrose residue. In studies of inulin decomposing enzymes, inulinase [EC 3.2.1.7] from molds and yeasts were reported in the past. A new type of inulin decomposing enzyme produced by *Arthrobacter ureafaciens* was discovered[1] (Uchiyama et al. 1973). The enzyme converted inulin into an 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]. Afterwards, there have been several reports on the inulin fructotransferase (DFA Ⅲ-producing) from *Arthrobacter* species[2, 3, 4, 5] . Kang et al.[6] reported on the enzyme from *Bacillus* sp.

Subsequently, we found another type of inulin decomposing enzyme[7] (Seki et al. 1989) from *Arthrobacter globiformis* S14-3. The enzyme converted inulin into oligosaccharide DFA Ⅰ (di-D-fructofuranose 1,2 ' 2,1 'dianhydride) and a small amount of other oligosaccharides. The enzyme was designated as inulin fructotransferase (DFA Ⅰ-producing) [EC 2.4.1.200]. On the enzymes producing DFA Ⅰ from inulin, an enzyme from *Streptomyces* sp.[8] was reported. Ueda et al.[9] (1994) reported on an enzyme from *Arthrobacter* sp. And we reported on the enzyme from *Arthrobacter ureafaciens* A51-1[10] .

In Japan, about 600,000 tons of sucrose is produced from sugar beet, annually. But, the sucrose consumption is decreasing in this market, and it is necessary to consider alternative crops for sugar beet. Chicory is a leading candidate alternative crop to sugar beet. The DFA Ⅰ, which can produce from chicory, has half the sweetness of sucrose, and has a potential as a new type of a low calorie sweetener.

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Recently, we isolated a microorganism, strain B69-5, which produced an inulin fructotransferase (DFA Ⅰ-producing) in the culture supernatant with high activity. Through taxonomical studies the microorganism was identified as *Arthrobacter* sp. B65-9. In this paper we describe the purification and properties of the enzyme.

## Materials and methods

### Identification of microorganism

For the extraction of the chromosomal DNA, Instagene matrix kit (Bio rad Co. Ltd., USA) was used. For the amplification of 16S r DNA Geneamp PCR system 9600 (Applied biosystems Co. Ltd., USA) was used. For the molecular genealogical analysis, Dnasis pro software (Hitachi software engineering Co. Ltd., Japan) was used.

### Cultivation of microorganism

*Arthrobacter* sp. B69-5 was pre-cultured in a 500 ml shaking flask at 30℃, for 24 h. The pre-culture medium was 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℃, for 24h. After the cultivation, the cells were removed by centrifugation (8000 x g, 30 min) and the supernatant was dialyzed against 10 mM Tris-HCl buffer, pH 8.5. The dialyzed solution was used as a crude enzyme solution.

### Standard assay methods

For the measurement of the enzyme activity, 0.1M citrate buffer, pH5.5 (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 45℃, for 30 min, and the reaction was stopped by heating at 100℃, for 7 min. The DFA Ⅰ produced was determined by HPLC (column, Shim-pack CLC ODS, 4.6 mm x 25 cm (Shimadzu Co. Ltd., Kyoto); mobile phase, water; detector, RI detector). One unit of the enzyme was defined as which can produce 1 μ mole of DFA Ⅰ per min at pH5.5 and 45℃. The protein concentrations were determined by the method of Lowry et al.[11] (1951) using bovine serum albumin as a standard.

### Purification of enzyme

The crude enzyme solution was applied on a column of DEAE- Toyopearl 650M (2.5 cm x 17 cm) equilibrated with 10 mM Tris-HCl buffer, pH8.5. The elution was performed with linear, 0 to 0.5M NaCl gradient in the same buffer. Fractions showing the enzyme activity were pooled and dialyzed against 10 mM Tris-HCl, pH8.0, containing 110g / l of ammonium sulfate. The dialyzed solution was applied on a column of butyl Toyopearl 650M (1.5 cm x 12 cm) equilibrated with 10 mM Tris-HCl buffer, pH8.0, containing 110 g / l of ammonium sulfate. The elution was performed with linear, 110 to 0 g / l ammonium sulfate gradient in the same buffer. The fractions showing the enzyme activity were pooled and dialyzed against 10mM Tris-HCl buffer, pH 8.5. The dialyzed solution was used as purified enzyme solution.

### 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-10L, 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 0.3 M NaCl; detection, UV280 nm).

### 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).

### Preparation of reaction products

For the preparation of the reaction products after the exhaustive enzyme reaction, 0.1 M citrate buffer, pH5.5 (0.5 ml), the purified enzyme solution (5 ml, 1.0 U/ml) and 5% inulin (10 ml) were mixed. The enzyme reaction was performed at 45℃, for 21 h and reaction was stopped by heating at 100℃ for 7 min. After cooling, the reaction mixture was analyzed by a paper chromatography. The paper chromatography was performed at 37℃ using Toyo No.50 filter paper (Advantech 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. The standard DFA I was prepared using the enzyme from *Arthrobacter ureafaciens* A51-1 [10].

## Results and discussion

### Microorganism and identification

Table 1 shows the taxonomic characteristics of the strain B69-5. From these results, the strain B69-5 was estimated to be a coryneform bacterium. Figure 1 shows the results of molecular genealogical analysis of 16S r DNAs. The strain B69-5 formed a single branch and did not form a same cluster with any type of strain of known *Arthrobacters*. From these results, the strain was identified as *Arthrobacter* sp. B69-5.

**Table 1. The taxonomic characteristics of strain B69-5**

| | |
|---|---|
| Shape and size | rod: 0.8 x 2.0-3.0 μm (12h) |
| Gram staining | positive |
| Spore formation | − |
| Motility | − |
| Catalase | + |
| Oxidase | − |
| Alkaline phosphatase | − |
| β-galactosidase | + |
| α-glucosidase | + |
| Reduction of nitrate | − |
| Urease | − |
| Gelatin hydrolysis | + |

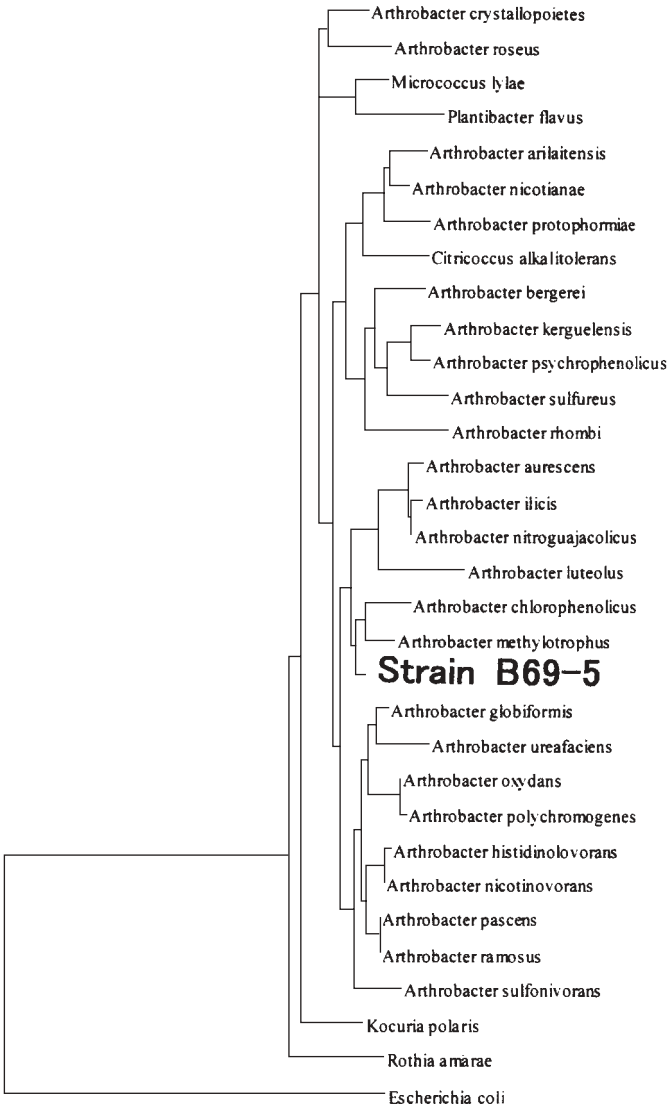



**Fig .1 Molecular genealogical analysis of 16S rDNA.**

**Table 2. Purification of inulin fructotransferase (DFA I -producing) from *Arthrobacter* sp. B69-5.**

| Step | Total protein (mg) | Total activity (units) | Specific activity (U/mg) | purification (fold) | Recovery (%) |
|---|---|---|---|---|---|
| Crude enzyme | 88.0 | 1040 | 11.8 | 1 | 100 |
| EAE-Toyopearl | 6.80 | 328 | 48.2 | 4.08 | 31.5 |
| Butyl-Toyopearl | 1.68 | 186 | 111 | 9.41 | 17.9 |

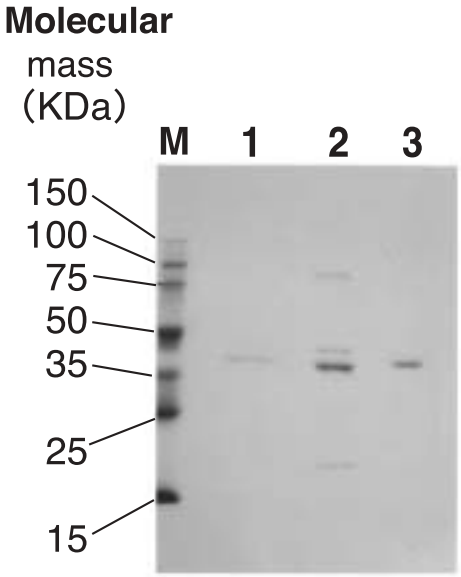



**Fig .2 SDS-PAGE of the intermediate fractions and the purified enzyme.**

Lane M, Molecular mass standard markers; lane 1, Crude enzyme; lane 2, DEAE-Toyopearl fraction; lane 3, Butyl Toyopearl fraction (purified enzyme).

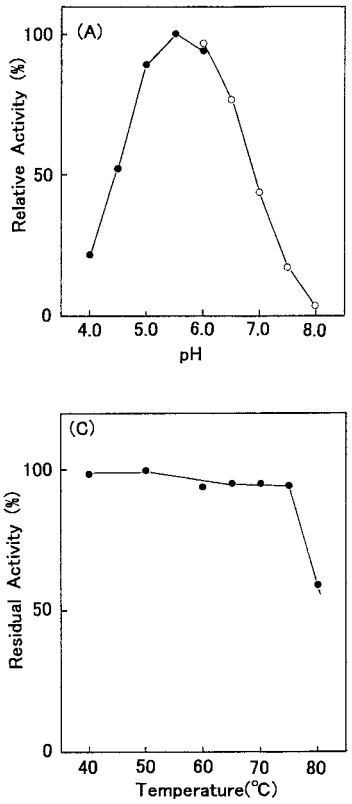



**Fig .3(A) Effect of pH on the enzyme activity; (●), Citrate buffer; (○), Phosphate buffer.**

**Fig. 3(B) Effect of temperature on the enzyme activity.**

**Fig. 3(C) Thermal stability of the enzyme.**

## Purification and characterization of enzyme

Table 2 shows a summary of the enzyme purification. The enzyme was purified by 9.41 -fold with a yield of 17.9 % by a DEAE-Toyopearl chromatography and a butyl Toyopearl chromatography. The purified enzyme was analyzed by SDS-PAGE. As shown in Fig.2, it gave a single band.

The effect of pH on the enzyme activity was investigated in the pH range 4.0-8.0 at 45℃. As shown in Fig. 3(A), maximum activity was obtained at pH5.5. The enzyme reaction was performed in the range 30-70℃ at pH5.5. As shown in Fig. 3(B), maximum activity was obtained at 45℃. The enzyme solution was heated at various temperatures for 20 min at pH5.5, then residual activities were measured at pH5.5 and 45 ℃. As shown in Fig. 3(C), the enzyme was stable up to 75℃, but it lost 40 % of activity at 80℃. This thermal stability is next to that of *A. ureafaciens* A51-1[10] (80 ℃).

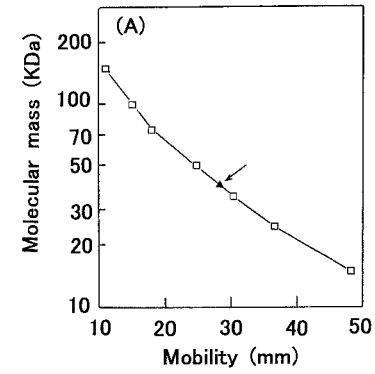
Fig. 4(A) shows plots of logarithmic molecular mass of the enzyme vs. protein mobility on SDS-PAGE. The molecular mass of the enzyme was estimated to be 40 000. Fig. 4(B) shows the result of molecular mass estimation by gel filtration with TSK-gel G3000SWXL. The molecular mass was estimated to be 42 000. From these results, the enzyme was considered to be a monomer. Table 3 summarizes the comparison of some properties of inulin fructotransferase (DFA Ⅰ-producing) from different microorganisms.

## N-terminal amino acid sequence

The N-terminal amino acid sequence (20 amino acid residues) was determined as ANTVYDVTTWS-GATISPYVD. So far, N-terminal amino acid sequences of inulin fructotransferase (DFA Ⅰ -producing) from *Arthrobacter globiformis* S14-3[7] and *Arthrobacter ureafaciens* A51-1[10] have been reported . The amino acid sequence of N-terminal region of the enzyme from the strain B69-5 was identical with that of from *A. globiformis* S14-3 and *A. ureafaciens* A51-1. However, as shown in Table 3, the properties of the three enzymes were different. It was estimated that the differences arose from the difference of the amino acid sequences of internal to C-terminal region of the three enzymes.

## Reaction products

The exhaustive enzyme reaction mixture, was analyzed by paper chromatography as mentioned in **materials and methods**. The Rf value for the main reaction product and two residual oligosaccharides (minor products) were 0.92, 0.44, and 0.35, respectively. The Rf values for the standard materials (DFA Ⅰ, $GF_2$ (1-kestose), $GF_3$ (nystose), $GF_4$ (fructofuranosyl nystose) were 0.92, 0.54, 0.44, and 0.34, respectively (data not shown). Therefore, the residual oligosaccharides (minor products) were estimated to be $GF_3$, and $GF_4$. This minor products were same as those of *A. ureafaciens* A51-1[10].



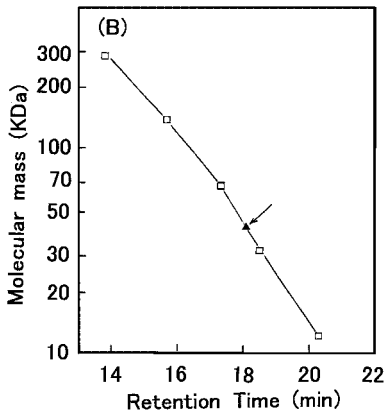




**Fig. 4(A) Estimation of the molecular mass by SDS-PAGE. Standard marker proteins, Takara perfect protein markers (150; 100; 75; 50; 35; 25; 15 kDa).**

**Fig. 4(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); cytchrome c (12.4 kDa).**

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

| Microorganism | Optimum pH | Optimum Temp. (℃) | Heat Stability (℃) | Molecular mass (kDa) SDS-PAGE | Molecular mass (kDa) gel filtration | References |
|---|---|---|---|---|---|---|
| *Arthrobacter* sp. B69-5 | 5.5 | 45 | 75 | 40 | 42 | This work |
| *A.globiformis* S14-3 | 6.0 | 40 | 70 | 39 | 46 | Seki et al. (1989) |
| *Arthrobacter* sp. MCI-2493 | 6.0 | 40 | 70 | 40 | 40 | Ueda et al.. (1991) |
| *A.ureafaciens* A51-1 | 5.5 | 45 | 80 | 44 | 79 | Haraguchi et al. (2003) |
| *Streptomyces* sp. MCI-2524 | 6.0 | 65 | 65 | 36 | 70 | Kushibe et al. (1993) |

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## *Arthrobacter* sp. B69-5のInulin fructotransferase (DFA I-producing)の精製と性質


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多糖類イヌリンからオリゴ糖DFAⅠを生じる酵素の生産菌が新たに分離された．分類学的な検討を行った結果，本菌株は*Arthrobacter* sp. B69-5と同定された．本菌株が培養上清中に生産する酵素をイオン交換クロマト，疎水クロマトにより電気泳動的に均一に精製した．本酵素の反応至適pHは5.5，反応至適温度は45℃であった．20分間の加熱に対して，本酵素は75℃まで安定であった．本酵素の分子量についてはSDS-PAGEから40kDa，ゲル濾過から42kDaという数字が得られ単量体酵素であると推察された．酵素蛋白のN-末端のアミノ酸配列を分析するとANTVYDVTTWSGATISPYVDという結果が得られた．