

## 技術報告

The gram-scale preparation of oligosaccharide DFA I using the enzyme from *Arthrobacter ureafaciens* A51-1.

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## Abstract

The method for the gram-scale preparation of oligosaccharide DFA I was developed, for the first time. Using the partially purified inulin fructotransferase (DFA I-producing) from *Arthrobacter ureafaciens* A51-1, the crude DFA I was prepared. With the baker's yeast treatment, ion exchange resin treatment and the gel filtrations by Toyopearl HW40S purified DFA I have prepared. With the method described in this paper, 2.5 g of purified DFA I was prepared from 10 g of inulin..

## 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 has previously been reported. A new type of inulin decomposing enzyme produced by *Arthrobacter ureafaciens* was discovered<sup>1)</sup> (Uchiyama *et al.* 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]. Since then, there have been several reports on the inulin fructotransferase (DFA III-producing) from *Arthrobacter* species<sup>2)~5)</sup>. Kang *et al.*<sup>6)</sup> reported on the enzyme from *Bacillus* sp.

Subsequently, we discovered another type of inulin decomposing enzyme<sup>7)</sup> (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]. In the enzymes producing DFA I from inulin, an enzyme from *Streptomyces* sp.<sup>8)</sup> was reported. Ueda *et al.* (1994) reported on an enzyme from *Arthrobacter* sp. and we reported on the enzyme from *Arthrobacter ureafaciens* A51-1<sup>10)</sup>.

In the previous reports<sup>7)~10)</sup> on the DFA I producing enzyme, the amount of the prepared DFA I was mg-scale. To elucidate the functions of the oligosaccharide DFA I by the experiment on animals, gram-scale amounts are needed. In this paper, we describe the method for preparation of DFA I of the amount of gram-scale, for the first time.

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## Materials and methods

### Cultivation of microorganism

*Arthrobacter ureafaciens* A51-1 was pre-cultured in 500 ml shaking flask (medium 100 ml) at 30°C, for 24h. The pre-culture medium was composed of 0.4 %  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ , 0.1 %  $\text{KH}_2\text{PO}_4$ , 0.1 %  $\text{NaNO}_3$ , 0.05 %  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.001 %  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.001%  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.05 % yeast extract (Difco), and 0.3% inulin at pH 7.0. The pre-culture was inoculated in a 5l 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 x g, 30 min) and the supernatant was dialyzed against 10 mM Tris-HCl buffer at pH 8.5. The dialyzed solution was used as a crude enzyme solution.

### Standard assay methods

For the measurement of the enzyme activity, 0.1 M citrate buffer at 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 50°C, for 30 min, and the reaction was stopped by heating at 100°C, 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  $\mu$  mole of DFA I per min at pH5.5 and 50°C. The protein concentrations were determined by the method of Lowry *et al.*<sup>11)</sup> (1951) using bovine serum albumin as a standard.

### Partial purification of enzyme

The crude enzyme solution was applied to a column of DEAE-Toyopearl 650 M (2.5 cm x 17 cm) equilibrated with 10 mM Tris-HCl buffer at pH8.5. The

elution was performed with linear, 0 to 0.5 M NaCl gradient in the same buffer. Fractions showing the enzyme activity were pooled. The obtained fraction was dialyzed against 10 mM Tris-HCl buffer at pH 8.5, and the dialyzed solution was used for the enzyme reaction.

### <sup>13</sup>C NMR spectrum

The <sup>13</sup>C NMR spectrum was recorded in D<sub>2</sub>O with an Avance 500 spectrometer (Bruker Co. Ltd., Germany) using 3-trimethylsilyl-1-propanesulfonic acid sodium salt (DSS) as an external standard.

## Results and discussion

### Partial purification of enzyme

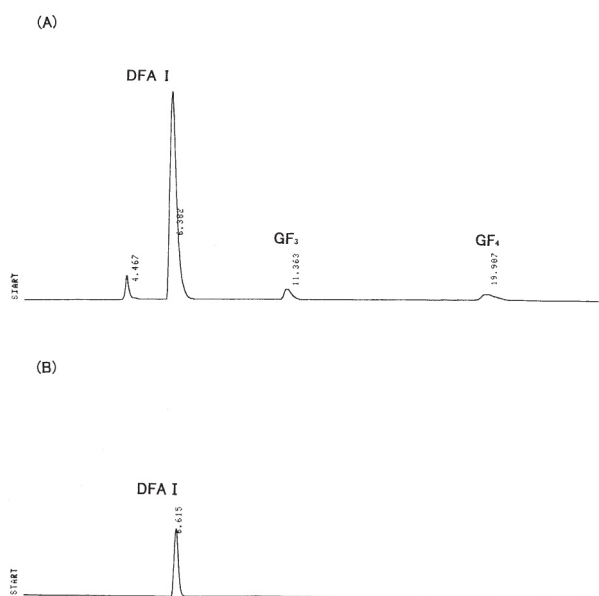
Table 1 shows the summary of the enzyme purification. With this process, the specific activity was increased about 4 times, and recovery of the activity was 31.0 %. The fraction obtained at this stage was used for the production of crude DFA I.

### Preparation of crude DFA I

The inulin (10 g) was dissolved in 100 ml of water with a warming by boiling water. After cooling, 2 ml of 0.1 M citrate buffer at pH 5.5 and 40 ml of the partially purified enzyme (2.7 U/ml) was added. The pH of the mixture was adjusted at 5.5. The enzyme reaction was performed at 50°C for 24 h. The reaction was stopped by heating at 100°C for 7 min. After cooling, the prepared crude DFA I solution was analyzed by HPLC (Shimpack CLC ODS). As shown in Fig 1 (A), the solution contains DFA I (main product) and the residual oligo-saccharides (GF<sub>3</sub> and GF<sub>4</sub>).

**Table 1 Partial purification of inulin fructotransferase (DFA I-producing)**

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)
Crude enzyme	520	99.3	5.24	100
DEAE-Toyopearl	161	7.92	20.3	31.0



**Fig 1 . Analysis of prepared DFA I**  
(A), Crude DFA I; (B), Purified DFA I.

## Purification of DFA I

### 1 ) Baker's yeast treatment.

A dry baker's yeast (0.2 g) was suspended in 20 ml of water. The suspension was centrifuged (8 000 x g, 30 min) and the precipitate was re-suspended in 10 ml of water. To remove the fermentable sugars by alcohol fermentation, the bakers yeast suspension (10 ml) was added to the crude DFA I solution. The suspension was incubated at 30°C for 4 h. After the incubation, the yeast cells were removed by centrifugation (8,000 x g, 30 min). The supernatant was heated at 100 °C for 7 min.

### 2) The ion exchange resin treatment.

After cooling, an ion exchange resin Amberlite MB-

3 (5 g) was added to the reaction mixture, and rested for 4 h. The resin was removed by a filter paper (Advantec No. 2), and the filtrate was concentrated by freeze drying.

### 3) The gel filtration (1<sup>st</sup>)

The freeze dried sample was dissolved in 8 ml of water (sample *a*). A part of the sample *a* (2 ml) was applied on a column of Toyopearl HW40S (5.0 cm x 20 cm, Tosoh Co. Ltd.) equilibrated with water. The elution was performed by water and the fractions containing DFA I were pooled. This gel filtration (1<sup>st</sup>) was repeated 8 times and the all of the sample *a* was used. The fractions obtained by the gel filtrations (1<sup>st</sup>) were combined.

### 4) The gel filtration (2<sup>nd</sup>)

The combined fraction was concentrated by freeze drying. The freeze dried sample was dissolved in 5 ml of water (sample *β*). A part of the sample *β* (2 ml) was applied on a column of Toyopearl HW40S (5.0 cm x 20 cm) equilibrated with water. The elution was performed by water and the fractions containing DFA I were pooled. The gel filtration was repeated 5 times and all of the sample *β* was used. The fractions obtained at this step were combined.

## Analysis of purified fraction

The obtained DFA I fraction was analyzed by HPLC. As shown in Fig. 1(B), the obtained fraction contained pure DFA I. The amount of purified DFA I obtained was 2.5 g (The amount was determined by HPLC.). A part of the purified fraction was concentrated by freeze drying and analyzed by <sup>13</sup>C NMR. As shown in Table 2, the chemical shifts of the main

**Table 2 <sup>13</sup>C NMR chemical shifts of main reaction product and DFA I**

Assignment carbon atom number	Chemical shifts (ppm) of <sup>13</sup> C-NMR of			
	Main reaction produc		Standard DFA I (ref.10)	
	( <i>a</i> )	( <i>β</i> )	( <i>a</i> )	( <i>β</i> )
1	65.3	63.9	65.3	63.8
2	105.2	101.5	105.1	101.5
3	84.5	86.2	84.5	86.2
4	79.6	77.2	79.6	77.2
5	83.9	80.4	83.9	80.4
6	64.4	65.2	64.4	65.2

reaction product agreed very closely with those of the standard DFA I.

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## *Arthrobacter ureafaciens* の酵素によるグラムスケールでのオリゴ糖 DFA I の調

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動物試験などによる機能性解明の実験に用いる目的で、グラムスケールでのオリゴ糖 DFA I の調製法を初めて確立した。 *A. ureafaciens* A51-1 由来の DFA I 合成酵素を部分精製し、粗製 DFA I の調製を行った。ここからパン酵母によるアルコール発酵処理、イオン交換

樹脂処理、トヨパール HW40S カラムによるゲル濾過を行うことにより、精製 DFA I オリゴ糖の標品を得た。原料であるイヌリン 10 g から精製 DFA I 2.5g を回収することができた。