研究ノート

Inulin Fructotransferase (DFA III-producing) From *Arthrobacter nicotinovorans* K-9

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Abstract

An inulin fructotransferase (DFA III-producing) [EC 2.4.1.93] from *Arthrobacter nicotinovorans* K-9 was purified and characterized. The enzyme was purified 35.7-fold from the culture supernatant of the microorganism with a yield of 12.1 %. The enzyme showed maximum activity at 60℃ and pH 6.0, and its activity was stable up to 70℃ after 30 min of heat treatment. Its molecular mass was estimated to be 40 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and 70 kDa by gel filtration, and the enzyme was considered to be a dimer.

Key words: *Arthrobacter*, DFA III (difructose dianhydride III), inulin, enzyme, purification

Introduction

Inulin is a polysaccharide found in chicory, *Dahlia*, Jerusalem artichoke, and other plants. Its chemical structure is a β-2, 1- linked fructose polymer terminated by a sucrose residue. Inulinase [EC 3.2.1.7] is an inulin-decom posing enzyme, which is present in molds and yeast. In addition a novel inulin-decomposing enzyme was found to be produced by *Arthrobacter ureafaciens* [1]. The enzyme converts inulin into a di-D-fructofuranose 1,2':2, 3' dianhydride (DFA III) oligosaccharide and a small number of other oligosaccharides, and it was designated as inulin fructotransferase (DFA III-producing) [EC 2.4.1.93]. Subsequently, there have been several reports on the presence of inulin fructotransferase in *Arthrobacter* species [2)-5)] and Kang et al.[6)] reported that the enzyme is found in *Bacillus* sp. We have reported that the enzyme is also found in *Leifsonia* sp. [7)].

The DFA III is half as sweet as sucrose and it accelerates the assimilation of minerals (i.e. calcium and iron) from intestine[8)]. Therefore, DFA III has potential to be used for osteoporosis and anemia caused by iron deficiency. The industrial production of DFA III started in Japan in 2004. DFA III containing commodities are now commercially available at drugstores and convenience stores in Japan. Thus, inulin fructotransferase (DFA III-producing) is an industrially important enzyme, even though its price is very high (¥50,000/ kg). The high cost is partly because of the low productivity of the enzyme producing microorganisms, and hence microorganisms with of high enzyme activity are required.

We recently isolated a microorganism, strain K-9, which produced highly active inulin fructotransferase in the culture supernatant. Through taxonomic studies, the microorganism was identified as *Arthrobacter nicotino-*

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vorans. In this paper, we describe how we identified the microorganism, and also the purification methods and properties of the enzyme.

Materials and Methods

Microorganism identification

We isolated a microorganism, strain K-9, from a soil sample collected in Chiba prefecture, Japan. Genomic DNA extraction, rDNA amplification, and DNA sequencing were performed by NCIMB Japan, Co Ltd., Shizuoka, Japan. For the extraction of a genomic DNA, achromopeptidase (Wako, Pure Chemicals, Ltd., Japan) was used. The amplification of 16S rDNA was performed using PrimeSTAR HS DNA polymerase (Takara Bio Inc, Japan). The DNA sequencing was performed using an ABI PRISM 3130xl genetic analyzer system (Applied Biosystems, USA).

Cultivation of the microorganism

To generate a starter culture, 1 loop of the microorganism (strain K-9) was inoculated into 100 mL of medium (composed of 0.4 % (w/v) $Na_2HPO_4 \cdot 12H_2O$, 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) in a 500 mL shaking flask. The culture was incubated at 30℃ for 24 h, with reciprocal shaking. The starter culture 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 (8000g, 30 min) and the supernatant was used as a crude enzyme solution.

Standard assay methods

For measuring the enzyme activity, 0.5 mL of 0.1M citrate buffer, (pH 6.0), the enzyme solution (0.1 mL), deionized water (0.4 mL), and 2 % inulin (1.0 mL) were mixed. Enzyme reaction was performed at 60℃, for 30 min, and the reaction was stopped by heating at 100℃, for 7 min. The DFA III produced was determined by high-performance liquid chromatography (HPLC; 4.6 mm x 25 cm column; Shim-pack CLC ODS; Shimadzu Co Ltd, Kyoto, Japan; mobile phase, water; detector, refractive index detector). One unit of the enzyme was defined as the amount of the enzyme that could produce 1 μmole DFA III per min at 60℃ and pH 6.0. The protein concentrations were determined by the method of Lowry et al[9].

Purification of enzyme

The crude enzyme solution was dialyzed against, 10 mM Tris-HCl buffer, pH 8.0. The dialyzed enzyme solution was applied to a column of DEAE-Toyopearl 650M (2.5 cm x 20 cm, Tohsoh Co Ltd, Japan) equilibrated with 10 mM Tris-HCl buffer, pH 8.0. The elution was performed with a linear 0 to 0.5 M NaCl gradient in the same buffer. The fraction showing the enzyme activity was pooled and dialyzed against 10 mM Tris-HCl buffer, pH 8.0 containing ammonium sulfate (100 g/L). The dialyzed enzyme solution was applied to a column of Butyl-Toyopearl 650M (1.5 cm x 14 cm, Tohsoh Co Ltd, Japan) equilibrated with 10 mM Tris-HCl buffer, pH 8.0 containing an ammonium sulfate (100 g/L). The elution was performed with linear 100 to 0 g/L ammonium sulfate gradient in the same buffer. The fraction containing the enzyme activity was pooled and dialyzed against 10 mM Tris HCl buffer, pH 8.0. The dialyzed solution was applied to a column of Super Q Toyopearl (1.5 x 14 cm) equilibrated with 10 mM Tris-HCl buffer, pH 8.0. The elution was performed with 0 to 0.5 M NaCl gradient in the same buffer. The fraction containing the enzyme activity was pooled and used as the purified enzyme fraction.

Estimation of molecular mass

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

Amino acid sequencing

The purified enzyme was electrophoretically blotted on a PVDF membrane (Sequi-Blot, Bio-Rad Laboratories Co Ltd, USA). The amino acid sequence of the N-termi nal region of the enzyme was analyzed by automated Edman degradation with a G1005A Protein Sequencer (Hewlett Packard Co Ltd, USA).

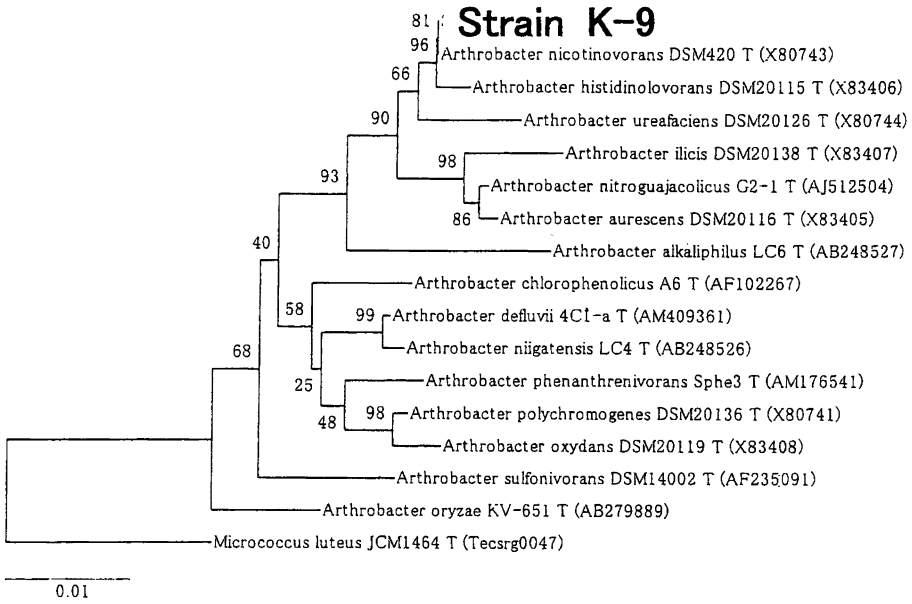

Fig. 1 Molecular genealogical analysis of the rDNA sequences of the microorganisms

The full-length of the 16S rDNA sequence was analyzed by Apollo 2.0 software (Techno Suruga Co. Ltd., Shizuoka). The small numbers at the junctions are the bootstrap values. The bar at the bottom left is a scale bar for genetic distance.

Preparation of reaction products from inulin

The reaction products were prepared by combining, 0.1 M citrate buffer, pH 6.0 (2.0 mL), purified enzyme solution (1.0 mL, 2.0 U/mL) and 4 % inulin (1.0 mL). The enzyme reaction was performed at 60℃ for 22 h and then, heated at 100℃ for 7 min to stop the reaction. After cooling, the reaction mixture was analyzed by a paper chromatography. Paper chromatography was performed at 37℃ 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.

Results and Discussion

Identification of the microorganism

Taxonomic studies were performed by NCIMB Japan, Shizuoka, Japan. The K-9 microorganism was a gram-positive, non-spore-forming aerobic bacterium, that was catalase positive and oxidase negative. From these results, strain K-9 was hypothesized to be a coryneform bacterium. The 16S rDNA sequence showed 99.8% homology with the sequence of *Arthrobacter nicotinovorans* (type strain). Molecular genealogical analysis of the 16S rDNA sequence showed that strain K-9 clustered with *Arthrobacter nicotinovorans* (Fig. 1). These results suggested that strain K-9 was *Arthrobacter nicotinovorans*.

Purification of enzyme

Table 1 shows the enzyme purification scheme. The enzyme was purified 35.7-fold with a yield of 12.1 % by a DEAE-Toyopearl, a Butyl Toyopearl and a Super Q-Toyopearl chromatography. The Super Q-Toyopearl fraction was analyzed by SDS-PAGE. As shown in Fig. 2, it gave a single band.

Effect of pH and temperature on enzyme activity

The effect of pH on the enzyme activity was investigated for the pH range of 4.0 to 8.0 at 60℃. As shown in Fig.3 (A), maximum activity was obtained at pH6.0. The enzyme reaction was performed in the range of 30℃ to 75℃ at pH 6.0. As shown in Fig. 3 (B), maximum activity was obtained at 60℃. Table 2 summarizes the properties of inulin fructotransferase from different microorganisms. In the industrial production of oligosaccharide DFA III, the enzyme from *Arthrobacter* sp. H65-7 is used. For increased production of DFA III, high specific activity of the enzyme is needed. We determined the specific activity of the enzyme of the enzyme from *A. nicotino-*

Table 1. Purification of the enzyme from *Arthrobacter nicotinovorans* K-9.

Step	Total activity (Units)	Total protein (mg)	Specific activity (U/mg protein)	Yield (%)
Crude enzyme	1650	78.2	21.1	100
DEAE-Toyopearl	882	6.60	134	53.5
Butyl-Toyopearl	397	1.01	393	24.1
Super Q-Toyopearl	199	0.264	754	12.1

Table 2. Comparison of inulin fructotransferases (DFA Ⅲ–producing)

Microorganisn	Optimum pH (℃)	Optimum temp.(℃)	Heat Stability	Molecular mass (kDa)		Ref.
				SDS-PAGE	Gel-filtration	
A. nicotinovorans K-9	6.0	60	70	40	70	This work
A. ureafaciens	6.0	50	50		80	(1)
A. globiformis C11-1	5.0	55	75	45	50	(2)
A. ilicis OKU-17B	5.5	60	70	27	50	(3)
Arthrobacter sp. H65-7	5.5	60	70	49	100	(4)
Arthrobacter sp. L68-1	5.5-6.0	55	80	43	73	(5)
Bacillus sp. snu-7	6.0	40	60	62		(6)
Leifsonia sp. T88-4	5.0	65	60	44	74	(7)

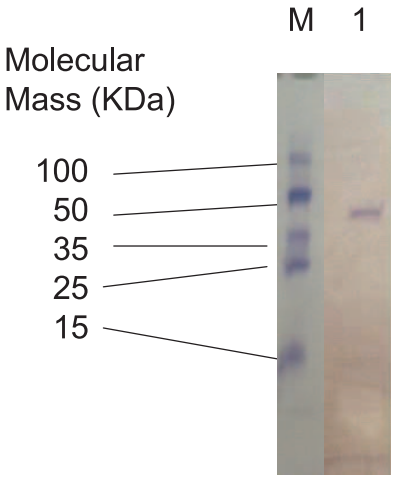

Fig. 2 The SDS-PAGE of the purified enzyme fraction

Lane M, Molecular mass standards; lane 1, Super Q- Toyopearl Fraction (0.1 μg protein/ lane). The protein bands were stained with Coomassie brilliant blue R250.

vorans K-9 to be 754 units/ mg protein (Table 1), while the known specific activity of the enzyme from *Arthrobacter* sp. H65-7 is 604 units/ mg protein4). Therefore, the specific activity of *A. nicotinovorans* K-9 is higher than that of the industrial strain.

Thermal stability

The enzyme solution was heated at various temperatures for 30 min at pH 6.0, and the residual activities were then measured at 60℃ and pH 6.0. As shown in Fig. 3 (C), the enzyme was stable up to 70℃, but it was inactivated at 75℃.

Molecular mass estimation

Based on the plots of logarithmic molecular mass of the enzyme vs. protein mobility determined by SDS-PAGE, the molecular mass of the enzyme was estimated to be 40 kDa. By gel filtration with TSK-gel G3000 SWXL, the molecular mass was estimated to be 70 kDa. On the basis of these results, the enzyme was considered to be a dimer.

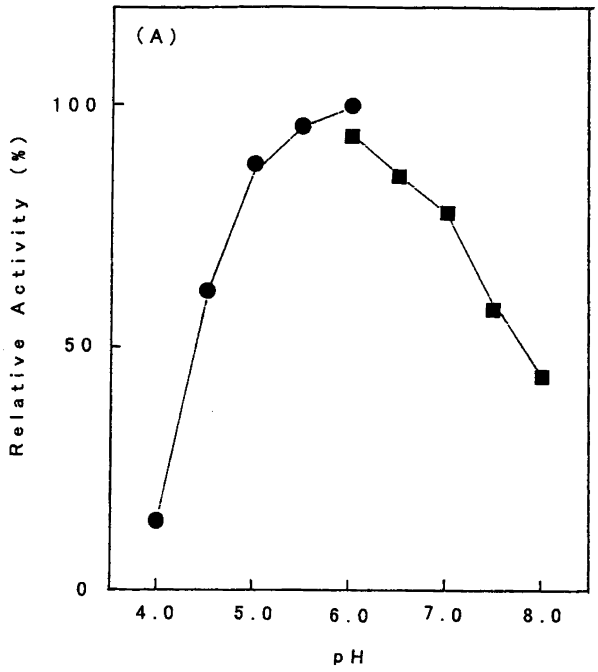

Fig. 3 (A) Effect of pH on the enzyme activity; (●), Citrate buffer; (■), Phosphate buffer.

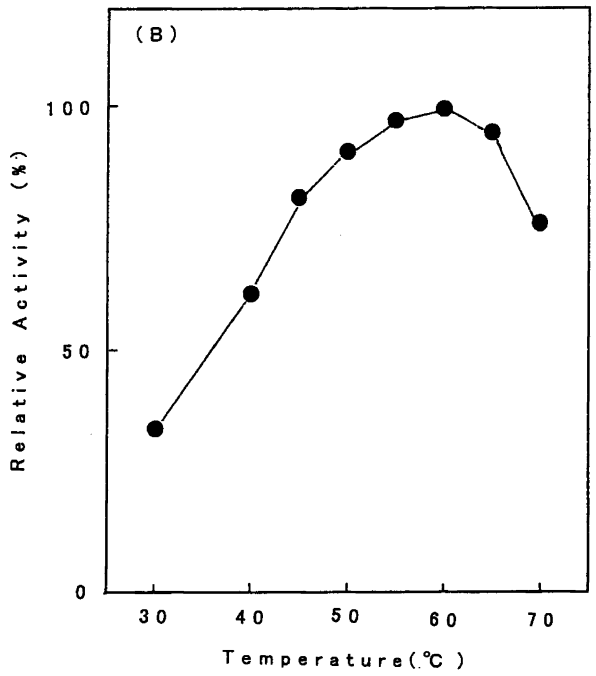

Fig. 3 (B) Effect of temperature on the enzyme activity.

N-terminal amino acid sequence

The N-terminal amino acid sequence of the enzyme was analyzed by a protein sequencer. However, the machine was unable to determine the N-terminal amino acid sequence.

Reaction products

The reaction mixtures of the enzyme from *A. nicotinovorans* K-9, were analyzed by a paper chromatography. The Rf values for the main reaction products and 2 residual oligosaccharides (minor products) were 0.98, 0.43 and 0.35, respectively. The R_f values for the standard materials (DFA Ⅲ, GF_2 (1-kestose), GF_3 (nystose), and GF_4 (fructosyl nystose)) were 0.98, 0.52, 0.42 and 0.35, respectively. Therefore, the residual oligosaccharides were believed to be GF_3, and GF_4.

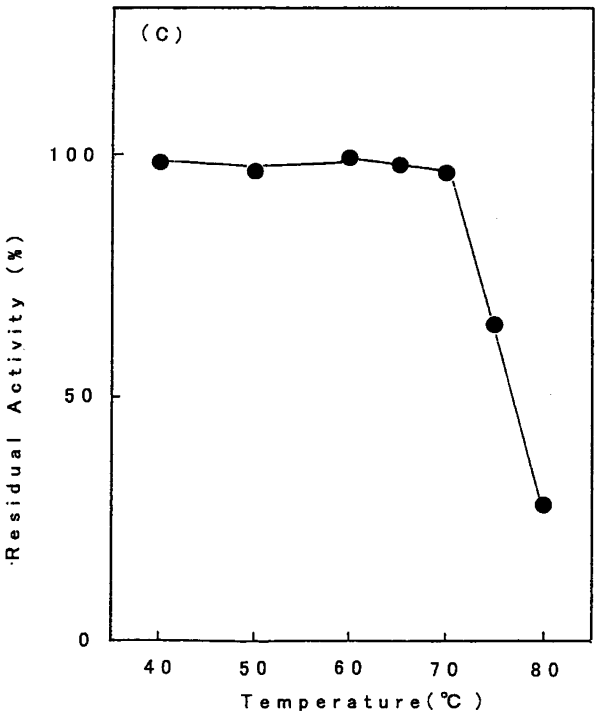

Fig. 3 (C) Thermal stability of the enzyme.

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Arthrobacter nicotinovorans K-9 の Inulin fructotransferase (DFA III-producing)

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要　約

新たに分離したDFA IIIオリゴ糖合成酵素生産菌K-9株について分類学的検討を行った結果，本菌は*Arthrobacter nicotinovorans*と同定された．本菌株のDFA III合成酵素を粗酵素液である培養上清からDEAE-トヨパール，Butyl-トヨパール，SuperQ-トヨパールによるクロマトグラフィーにより，電気泳動的に均一に精製した．本酵素の反応至適pHは6.0，反応至適温度は60℃であった．本酵素の耐熱性について検討すると70℃まで安定という結果が得られた．本酵素の分子量について検討するとSDS-電気泳動から40KDa，ゲル濾過から70KDaという値が得られた．このため本酵素はホモダイマーと推察される．