研究ノート

Inulin Fructotransferase (DFA I-producing) from *Arthrobacter* sp. H10-2

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Abstract

This manuscript reports the purification and characterization of an inulin fructotransferase (DFA I-producing) [EC 4.2.2.17] from *Arthrobacter* sp. H10-2. The enzyme was purified 35.6-fold from the culture supernatant of the microorganism with a 17.3 % yield. The enzyme exhibited maximal activity at pH 5.5 and 40 ℃; however, activity began to decrease after it was exposed to extreme conditions (*i.e.*, 75 ℃ for 30 min). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and gel filtration chromatography were used to estimate the molecular mass of the enzyme, which was determined as 45 kDa and 46 kDa, respectively. Therefore, it was predicted to be a monomer. Furthermore, we observed the production of the minor products of the enzyme reaction, GF_3 (nystose) and GF_4 (fructofuranosyl nystose).

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

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], which catalyzes the hydrolysis of inulin, is expressed in molds and yeasts. Following the discovery of inulinase, a new inulin-decomposing enzyme was discovered in *Arthrobacter ureafaciens*[1]. The enzyme converts inulin into a di-D-fructofuranose 1, 2': 2, 3' dianhydride (DFA III) oligosaccharide with additional oligosaccharide minor products formed. This enzyme was designated as inulin fructotransferase (DFA III-producing) [EC 4.2.2.18]. Subsequently, there have been several reports on the presence of inulin fructotransferase (DFA III-producing) in *Arthrobacter* species[2)-5)], and Kang *et al*.[6] reported that the enzyme is found in *Bacillus* sp. snu-7. We have reported that the enzyme is also found in *Leifsonia* sp.[7] T88-4.

We subsequently identified another type of inulin decomposing enzyme from *Arthrobacter globiformis* S14-3[8]. This enzyme converts inulin into di-D-fructofuranos 1, 2': 2, 1' dianhydride (DFA I) and a small amount of other oligosaccharides [EC 4.2.2.17]. The oligosaccharide DFA I exhibits half the sweetness of sucrose, and has potential as a new type of low-calorie sweetener, although the actual calorie count is not currently known. There are a few reports on inulin fructotransferase (DFA I-producing) from *Arthrobacter* species[9)-11)], and Kushibe *et al*. purified the enzyme from *Streptomyces* sp. MCI-2524[12].

Recently, we isolated a microorganism (strain H10-2) that produced an inulin fructotransferase (DFA I-producing). Through taxonomic analysis, this microorganism was identified as *Arthrobacter* sp. H10-2. In this paper, we describe the purification and properties of inulin fructotransferase (DFA I-producing) from this microorganism.

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Materials and Methods

Microorganism and identification

The *Arthrobacter* H10-2 strain was isolated from a soil sample collected in Aomori prefecture (Hirosaki city). Genomic DNA extraction, ribosomal DNA amplification, and DNA sequencing were performed by TechnoSuruga Laboratory Co. Ltd., Shizuoka, Japan. DNA sequencing was performed using an ABI PRISM 3130xl genetic analyzer system (Applied Biosystems, Co. Ltd.; USA).

Cultivation of the microorganism

To prepare a starter culture, the microorganism (strain H10-2) was inoculated into 100 mL of medium (composed of 0.4 % (w/v) $Na_2HPO_4 \cdot 12H_2O$, 0.1 % (w/v) KH_2PO_4, 0.1 % (w/v) $NaNO_3$, 0.05 % (w/v) $MgSO_4 \cdot 7H_2O$, 0.001 % (w/v) $CaCl_2 \cdot 2H_2O$, 0.001 % (w/v) $FeSO_4 \cdot 7H_2O$, 0.05 % (w/v) yeast extract (Difco), and 0.3 % (w/v) inulin, pH 7.0) in a 500-mL shaking flask..

The culture was incubated at 30 ℃ for 24 h with reciprocal shaking (120 rpm). The starter culture was inoculated in a 5-L Erlenmeyer flask containing 1 L of the same medium and incubated at 30 ℃ for 24 h with rotary shaking (120 rpm). After cultivation, the cells were removed by centrifugation (8000 × *g*, 30 min) and the supernatant was used as a crude enzyme solution.

Standard assay method

To measure the enzyme activity, 0.1 M citrate buffer, pH 5.5 (0.5 mL), the enzyme solution (0.2 mL), water (0.3 mL), and 2 % inulin (1.0 mL) were mixed. The enzyme reaction was performed at 40 ℃ for 30 min, and the reaction was stopped by heating at 100 ℃ for 7 min. The DFA I produced in the reaction was detected by high-performance liquid chromatography (HPLC; column, Shim-pack CLC ODS, 6.0 mm × 15 cm; Shimadzu Co. Ltd.; mobile phase, water; detector, refractive index detector). One unit of the enzyme was defined as the amount of the enzyme that could produce 1 μmol DFA I per minute at 40 ℃ and pH 5.5. The protein concentrations were determined by employing the method of Lowry *et al* [13].

Purification of enzyme

The crude enzyme solution was dialyzed against 10 mM Tris-HCl buffer, pH 8.5. The dialyzed solution was applied to a column of DEAE-Toyopearl 650M (2.5 × 20 cm; Tosoh Co. Ltd.; Japan) equilibrated with 10 mM Tris-HCl buffer, pH 8.5. The elution was performed with a linear 0 to 0.5 M NaCl gradient in the same buffer. The fractions exhibiting enzymatic activity were 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 × 12 cm; Tosoh Co. Ltd.) equilibrated with 10 mM Tris-HCl buffer, pH 8.0, containing ammonium sulfate (100 g/L). The elution was performed with a linear 100 to 0 g/L ammonium sulfate gradient in the same buffer. The fractions exhibiting enzyme activity were pooled and dialyzed against 10 mM phosphate buffer, pH 8.0. The dialyzed solution was applied to a column of Super Q-Toyopearl 650M (1.5 × 14 cm; Tosoh Co. Ltd.) equilibrated with 10 mM phosphate buffer, pH 8.0. The elution was performed with a 0 to 0.5 M NaCl gradient in the same buffer. The fractions displaying enzymatic activity were pooled and used as the purified enzyme solution.

Estimation of molecular mass

The molecular mass of the enzyme was estimated by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a ready-made gel (Pagel NPU-12.5L; Atto, Co. Ltd.; Japan). In addition, the molecular mass of the enzyme was estimated by gel filtration on an HPLC column (TSK-gel G3000SWXL; Tohsoh Co. Ltd.,; mobile phase, 100 mM sodium phosphate buffer, pH 7.0, containing 0.5 M NaCl; flow rate, 0.7 mL/ min; detection, UV 280 nm).

Amino acid sequencing

The purified enzyme was electrophoretically blotted on a polyvinyldifluoride membrane (Sequi-Blot; Bio-Rad Laboratories Co. Ltd.; USA). The amino acid sequence of the N-terminal region of the enzyme was analyzed by conducting automated Edman degradation with a G1005 Protein Sequencer (Hewlett Packard Co. Ltd.; USA).

Long-duration enzyme reaction

Citrate buffer, (0.1 M, pH 5.5, 0.2 mL), the enzyme solution (0.4 U/mL, 1 mL), and 4 % inulin (1 mL) were mixed and incubated at 40 ℃ for 23 h. The reaction was

stopped by heating to 100 ℃ for 7 min. After cooling, the reaction mixture was analyzed by paper chromatography.

Paper chromatography

Paper chromatography was performed at 37 ℃ using Toyo No.50 filter paper (Advantech Co. Ltd., Japan) with a solvent system of n-butyl alcohol: pyridine: water (3: 2: 2, by volume). The chromatogram was irrigated twice, and the spots of the reaction products were revealed with a resorcinol-HCl reagent.

Results and Discussion

Identification of the microorganism.

Taxonomic analyses were performed by TechnoSuruga Laboratory Co. Ltd., Shizuoka, Japan. The strain H10-2 was a gram-positive, non-spore-forming, aerobic bacterium; it was catalase-positive and oxidase-negative. Based on these results, strain H10-2 was hypothesized to be a coryneform bacterium. The 16S rDNA sequence showed 98.6 % homology with that of *Arthrobacter ramosus* (type strain), although the molecular genealogical analysis of the 16S rDNA sequence illustrated that strain H10-2 did not cluster with any *Arthrobacter* species strain (data not shown). Hence, the strain H10-2 was identified as *Arthrobacter* sp. H10-2.

Purification of the enzyme

Table 1 depicts the enzyme purification scheme. The enzyme was purified 35.6-fold with a yield of 17.3 % by using a DEAE-Toyopearl, a Butyl-Toyopearl, and a Super Q-Toyopearl chromatography system. The Super Q-Toyopearl fraction was analyzed by SDS-PAGE. As shown in Fig. 1, it purified to a single band.

Molecular mass estimation

Based on the SDS-PAGE results, the molecular mass of the enzyme was estimated to be 45 kDa (Fig. 1). Similarly, the gel filtration results showed its mass to be 46 kDa (Fig. 2). Given these data, the enzyme was estimated to be a monomer.

Effect of pH and temperature on enzymatic activity

We measured the pH-dependence of enzymatic activity from pH 4.0 to 8.0 at 40 ℃. As shown in Fig. 3 (A), maximal activity was observed at pH 5.5. The catalytic reaction was performed in the range of 30−70 ℃ at pH 5.5. As shown in Fig. 3 (B), maximal activity of the enzyme was observed at 40 ℃.

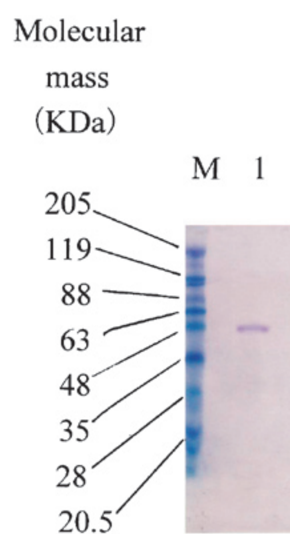

Fig. 1 SDS-PAGE of the purified enzyme fraction. Lane M, molecular mass standard markers. Lane 1, Super Q-Toyopearl fraction (purified enzyme).

Table 1. Purification of the enzyme from *Arthrobacter* sp. H10-2

Step	Total activity (units)	Total protein (mg)	Specific activity (U/mg protein)	Yield (%)
Crude enzyme	288	88.4	3.25	100
DEAE-Toyopearl	123	11.5	10.7	43.1
Butyl-Toyopearl	57.4	1.74	32.9	20.1
Super Q-Toyopearl	49.9	0.43	116	17.3

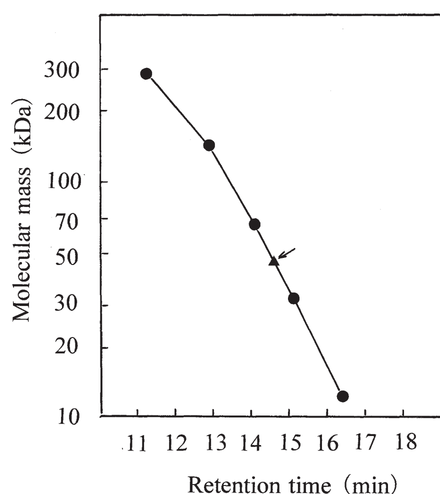

Fig. 2 Estimation of molecular mass by gel filtration chromatography retention time.

Thermal stability

The thermal stability of the heat-treated enzyme (30 min) was investigated at pH 5.5. As shown in Fig. 3 (C), the enzyme was stable up to 75 ℃. Table 2 compares the properties of inulin fructotransferase (DFA I-producing). The thermostability of the enzyme from *Arthrobacter* sp. H10-2 is just below that of the enzyme from *A. ureafaciens* A51-1[10], which exhibits the highest thermostability reported to date (80 ℃). Since the reaction time for large-scale industrial applications is rather long, the thermostability of the enzyme is very important. This enzyme possesses the highest thermostability among the monomeric inulin fructotransferase (DFA I-producing) enzymes (Table 2).

N-terminal amino acid sequence

Using a peptide sequencer, the N-terminal amino acid sequence was determined. The first and second amino acids were leucine and alanine, respectively. The third amino acid and subsequent sequence could not be determined. This indicated that the enzyme protein was either chemically modified or that the third amino acid was modified during the Edman degradation process.

Reaction products

The reaction mixture was analyzed by paper chromatography after the long-duration reaction, as described in **Materials and Methods**. The R_f values for the main reaction product and the two residual oligosaccharides (minor products) were 0.83, 0.26, and 0.17, respectively. The R_f values for the standard materials DFA I, GF_2 (1-kestose), GF_3 (nystose), and GF_4 (fructofuranosyl nystose) were 0.84, 0.38, 0.27, and 0.18, respectively. Therefore, we concluded that the residual oligosaccharides (minor products) were indeed GF_3 and GF_4, the same minor products produced by the enzyme from *Arthrobacter* sp. MCI2493[9].

Conclusion

We here reported the purification and characterization of inulin fructotransferase (DFA I-producing) from *Arthrobacter* sp. H10-2. Although the enzyme was stable up to 75 ℃, it exhibited maximal activity at 40 ℃ (pH 5.5). SDS-PAGE and gel filtration chromatography showed the molecular mass of the enzyme to be 45 kDa and 46 kDa, respectively, which is indicative of a monomer. The thermostability and specific activity of the enzyme were slightly lower in comparison to those from *Arthrobacter ureafaciens* A51-1[10].

References

1) Uchiyama, T., Niwa, S., & Tanaka, K. (1993) Purification and properties of *Arthrobacter ureafaciens* inulase II. *Biochim. Biophys. Acta*, **315**, 412-420.
2) Haraguchi, K., Kishimoto, M., Seki, K., Hayashi, K., Kobayashi, S., & Kainuma, K. (1988) Purification and properties of inulin fructotransferase (depolymerizing) from *Arthrobacter globiformis* C11-1. *Agric. Biol. Chem.*, **52**, 291-292.
3) Kawamura, M., Takahashi, S., & Uchiyama, T. (1988) Purification and some properties of inulin fructotransferase (depolymerizing) from *Arthrobacter ilicis., Agric. Biol. Chem.*, **52**, 3209-3210.
4) Yokota, A., Enomoto, K., & Tomita, F., (1991) Purification and properties of an inulin fructotransferase (depolymerizing) from *Arthrobacter* sp. H65-7. *J. Ferment. Bioeng.*, **72**, 262-265.
5) Haraguchi, K., Yoshida, M., & Ohtsubo, K. (2005) Thermostable inulin fructotransferase (DFA III-producing) from *Arthrobacter* sp. L68-1, *Carbohydr. Polym.*, **59**, 411-416.

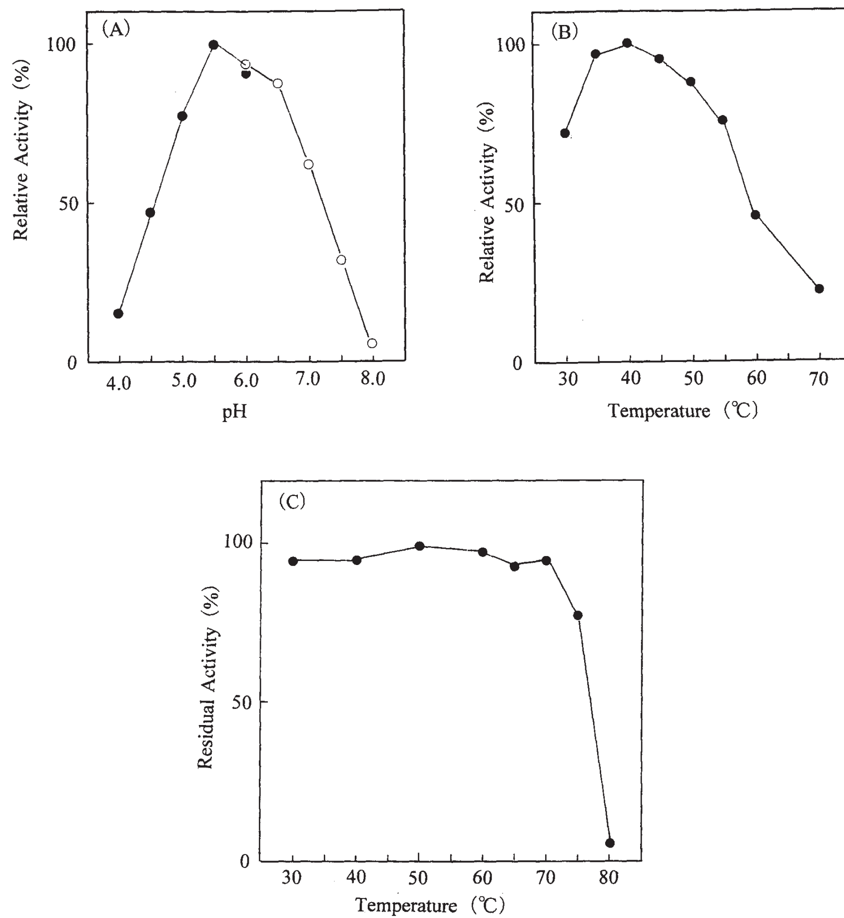

Fig. 3 (A) Effect of pH on enzymatic activity. ●; Citrate buffer, ○; Phosphate buffer.
(B) Effect of temperature on enzymatic activity.
(C) Thermostability of the enzyme.

Table 2. Comparison of properties of inulin fructotransferase (DFAI-producing)

Microorganism	Optimum pH	Optimum temperature (℃)	Thermo-stability (℃)	Molecular mass (KDa)		Specific activity (U/mg)	References
				SDS-PAGE	Gel filtration		
Arthrobacter sp. H10-2	5.5	40	75	45	46	116	This study
A. globiformis S14-3	6.0	40	70	39	46	81.5	(8)
Arthrobacter sp. MCI-2493	6.0	50	70	40	40	384	(9)
A. ureafaciens A51-1	5.5	45	80	38	61	172	(10)
A. pascens a62-1	5.5	45	75	37	60	127	(11)
Streptomyces sp. MCI-2524	6.0	55	65	36	70	256	(12)

6) Kang, S., Kim, W., Chang, Y., & Kim, S. (1998) Purification and properties of inulin fructotransferase (DFA III-Producing) from *Bacillus* sp. Snu-7. *Biosci. Biotech. Biochem.*, **62**, 629-631.

7) Haraguchi, K., Yoshida, M., & Ohtsubo, K. (2006) Inulin fructotransferase (DFA III-producing) from *Leifsonia* sp. T88-4. *Carbohydr. Polym.*, **66**, 75-80.

8) Seki, K., Haraguchi, K., Kishimoto, M., Kobayashi, S., & Kainuma, K. (1989) Purification and properties of a novel inulin fructotransferase (DFA I-producing) from *Arthrobacter globiformis* S14-3. *Agric. Biol. Chem.*, **53**, 2089-2094.

9) Ueda, M., Sashida, R., Morimoto, Y., & Ohkishi, H. (1994) Purification of inulin fructotransferase (DFA I-producing) from *Arthrobacter* sp. MCI2493 and production of DFAI from inulin by the enzyme. *Biosci. Biotech. Biochem.*, **58**, 574-575.

10) Haraguchi, K., Yoshida, M., & Ohtsubo, K. (2003) Purification and properties of a heat-stable inulin fructotransferase from *Arthrobacter ureafaciens*. *Biotech. Lett.*, **25**, 1049-1053.

11) Haraguchi, K., Yoshida, M., Yamanaka,T., & Ohtsubo, K (2003) Purification and characterization of a heat stable inulin fructotransferase (DFA I-producing) from *Arthrobacter pascens* a62-1., *Carbohydr. Polym.*, **53**, 501-503.

12) Kushibe, S., Sashida, R., Morimoto, Y., & Ohkishi, H. (1993) Purification and characterization of a di-D-fructofuranose 2', 1; 2, 1'-dianhydride producing enzyme from *Streptomyces* sp. MCI-2524. *Biosci. Biotech. Biochem.*, **57**, 2054-2058.

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

Arthrobacter sp. H10-2のDFA Ⅰオリゴ糖合成酵素

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要　旨

青森県弘前市の土壌から分離した細菌H10-2株は培養上清中にイヌリンからオリゴ糖DFA Iを生産する酵素を生産した．本菌株について分類学的な検討を行った結果，本菌株は*Arthrobacter*属の細菌と同定された．DFA Iオリゴ糖合成酵素を精製し，その性質を解析した．本酵素は粗酵素液からイオン交換クロマト, 疎水クロマトによって電気泳動的に単一バンドに精製された．精製の過程で比活性は約35.6倍に上昇し，17.3% の収率で精製酵素が得られた．本酵素の至適pHは5.5，至適温度は40℃であった．30分間の熱処理を行った場合の酵素の耐熱温度について解析した結果，本酵素は75℃まで安定的に活性を有していた．SDS-PAGEの結果分子量は約45 kDaであった．ゲル濾過の結果からは分子量は約46 kDaであった．これらの結果から本酵素は単量体と推察された．イヌリンに作用させた時の反応生成物を分析した結果，主産物としてDFA I，切れのこりの副産物としてフラクトオリゴ糖（GF_3，GF_4）が生成していることが示された．