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Short Communication

Purification and characterization of a heat stable inulin fructotransferase (DFA I-producing) from *Arthrobacter pascens* a62-1

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Abstract

An inulin fructotransferase (DFA I-producing) [EC 2.4.1.200] from *Arthrobacter pascens* a62-1 was purified and the properties of the enzyme were investigated. The enzyme was purified from culture supernatant of the microorganism 58.5 fold with a yield of 8.32% using Super Q Toyopearl chromatography and butyl Toyopearl chromatography. It showed maximum activity at pH 5.5 and 45 °C and was stable up to 75 °C. This heat stability was highest in the inulin fructotransferases (DFA I-producing) reported until now. The molecular mass of the enzyme was estimated to be 37,000 by SDS-PAGE and 60,000 by gel filtration, and was considered to be a dimer. The N-terminal amino acid sequence (20 amino acid residues) was determined as Ala-Asn-Thr-Val-Tyr-Asp-Val-Thr-Thr-Trp-Ser-Gly-Ala-Thr-Ile-Ser-Pro-Tyr-Val-Asp.

Keywords: Inulin; DFA I (difructose dianhydride I); *Arthrobacter*

1. 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 was reported in the early literature. A new type of inulin decomposing enzyme produced by *Arthrobacter ureafaciens* was discovered later (Uchiyama, Niwa, & Tanaka, 1973). This enzyme converted inulin into an oligosaccharide DFA III (di-D-fructofuranose 1,2′:2, 3′ dianhydride) and a small amount of other oligosaccharides and was designated as inulin fructotransferase (DFA III-producing) [EC 2.4.1.93]. Subsequently, there have been several reports on the inulin fructotransferase (DFA III-producing) from *Arthrobacter* species (Haraguchi, Kishimoto, Seki, Kobayashi, & Kainuma, 1988; Haraguchi, Yamanaka, & Ohtsubo, 2002; Kawamura, Takahashi, & Uchiyama, 1988; Yokota, Enomoto, & Tomita, 1991). Kang, Kim, Chang, and Kim (1998) reported on the enzyme from *Bacillus* sp.

We have reported another type of inulin decomposing enzyme (Seki, Haraguchi, Kishimoto, Kobayashi, & Kainuma, 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. (Kushibe, Sashida, Morimoto, & Ohkishi, 1993) and an enzyme from *Arthrobacter* sp. (Ueda, Sashida, Morimoto, & Ohkishi, 1994) were reported.

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

Recently, we isolated a microorganism, strain a62-1, which produced an inulin fructotransferase (DFA I-producing) in the culture supernatant. Through taxonomical

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studies, the microorganism was identified as *Arthrobacter pascens* a62-1. In this paper, we describe the purification and characterization of the enzyme.

2. Materials and methods

2.1. Cultivation of microorganism

For a pre-culture, *A. pascens* a62-1 was cultured in a 500 ml shaking flask at 30 °C for 24 h which contain a medium. The 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), 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 °C for 24 h. After the cultivation, the cells were removed by centrifugation (8000*g*, 30 min) and the supernatant was used as a crude enzyme solution.

2.2. Standard assay methods

For the measurement of 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 45 °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 × 25 cm (Shimadzu Co. Ltd, Kyoto); mobile phase, water; detector, RI detector). One unit of the enzyme was defined as that which can produce 1 μmol of DFA I per min at pH 5.5 and 45 °C. Protein concentrations were determined using the method of Lowry, Rosebrough, Farr, and Randall (1951) using bovine serum albumin as a standard.

2.3. Purification of enzyme

The crude enzyme solution was dialyzed against 10 mM Tris–HCl buffer, pH 8.5. The dialysate was applied to a column of Super Q Toyopearl 650M (2.5 cm × 17 cm) equilibrated with 10 mM Tris–HCl buffer, pH 8.5. The elution was performed with linear 0–0.5 M NaCl gradient in the same buffer. The fractions showing the enzyme activity were pooled and dialyzed against 10 mM sodium phosphate buffer, pH 8.0, containing 120 g/l of ammonium sulfate. The dialysate was applied to a column of butyl Toyopearl 650M (1.5 cm × 12 cm) equilibrated with 10 mM sodium phosphate buffer, pH 8.0, containing 120 g/l of ammonium sulfate. The dialysate was applied to column to butyl Toyopearl 650M (1.5 cm × 12 cm) equilibrated with 10 mM sodium phosphate buffer, pH 8.0, containing 120 g/l of ammonium sulfate. The elution was performed with linear 120–0 g/l ammonium sulfate gradient in the same buffer. The fractions showing the enzyme activity were pooled and dialyzed against water. The dialyzed solution was used as a purified enzyme solution.

2.4. 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 8.0, containing 0.3 M NaCl; detection, UV 280 nm).

2.5. 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 analyzed by automated Edman degradation with G1005A protein sequencer (Hewlett Packard Co. Ltd, USA).

2.6. Preparation of reaction products

For the preparation of the reaction products after the exhaustive enzyme reaction, 0.1 M citrate buffer, pH 5.5 (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 °C for 20 h and the reaction was stopped by heating at 100 °C for 7 min. After cooling, the reaction mixture was analyzed by a paper chromatography. The paper chromatography was performed at 37 °C 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. An ion exchange resin, Amberlite MB-3 (2.0 g, Organo Co. Ltd, Japan) was added to the rest of the reaction mixture and the suspension was kept 3 h at room temperature. After removing the resin by filtration, the reaction mixture was concentrated by freeze-drying. To separate the main reaction product, the freeze-dried sample was dissolved in 1 ml of water, and was applied on a column of Toyopearl HW40S (2.5 cm × 24 cm, Tosoh Co. Ltd, Japan) equilibrated with water. The elution was performed with water and the fractions containing the pure main

Table 1
Purification of the inulin fructotransferase (DFA I-producing) from *A. pascens* a62-1

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Crude enzyme	264	573	2.17	100
Super Q-Toyopearl	11.7	197	16.8	34.4
Butyl-Toyopearl	0.376	47.7	127	8.32

The purification was started from 3980 ml of the crude enzyme.

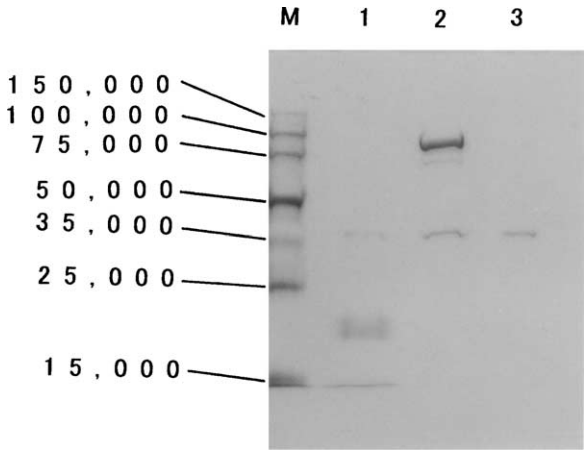

Fig. 1. SDS-PAGE of the intermediate fractions and the purified enzyme. Lane M, Molecular mass standard markers; land 1, crude enzyme; lane 2, Super Q Toyopearl fraction; lane 3, butyl Toyopearl fraction (purified enzyme).

product were pooled (the purity was checked by HPLC). The fraction was concentrated by freeze-drying and used as a sample of ^{13}C NMR. The ^{13}C NMR spectrum was recorded in D_2O with a BRX 600 spectrometer (Bruker Co. Ltd, Germany) using 3-trimethylsilyl-1-propanesulfonic acid sodium salt as a standard.

3. Results and discussion

3.1. Purification of enzyme

Table 1 shows a summary of the enzyme purification. The enzyme was purified 58.5 fold with a yield of 8.32% by Super Q-Toyopearl chromatography and butyl Toyopearl chromatography. The purified enzyme was analyzed by SDS-PAGE. As shown in Fig. 1, it gave a single band on SDS-PAGE.

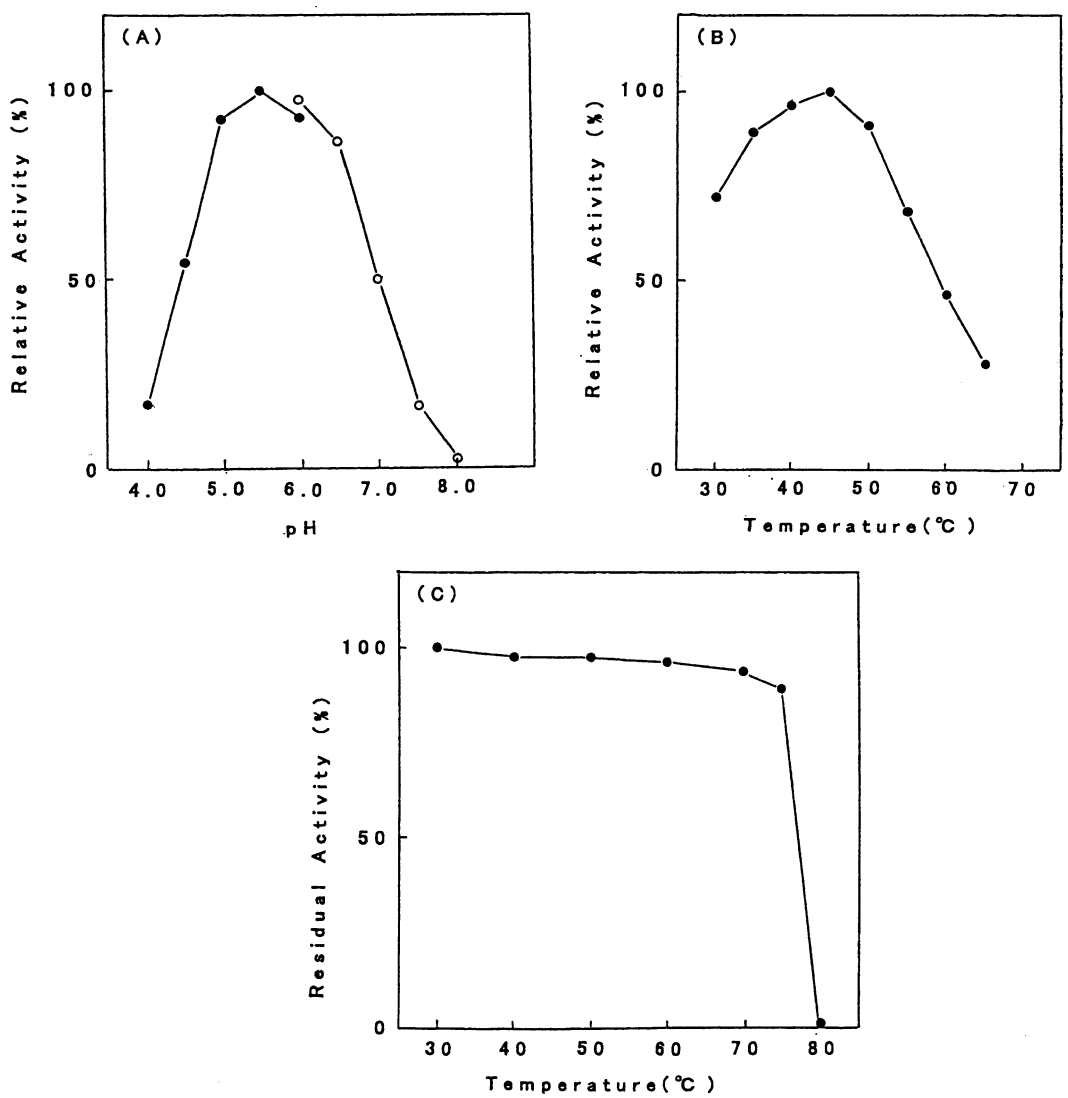

Fig. 2. (A) Effect of pH on the enzyme activity. (●), citrate buffer; (○), phosphate buffer. (B) Effect of temperature on the enzyme activity. (C) Thermal stability of the enzyme.

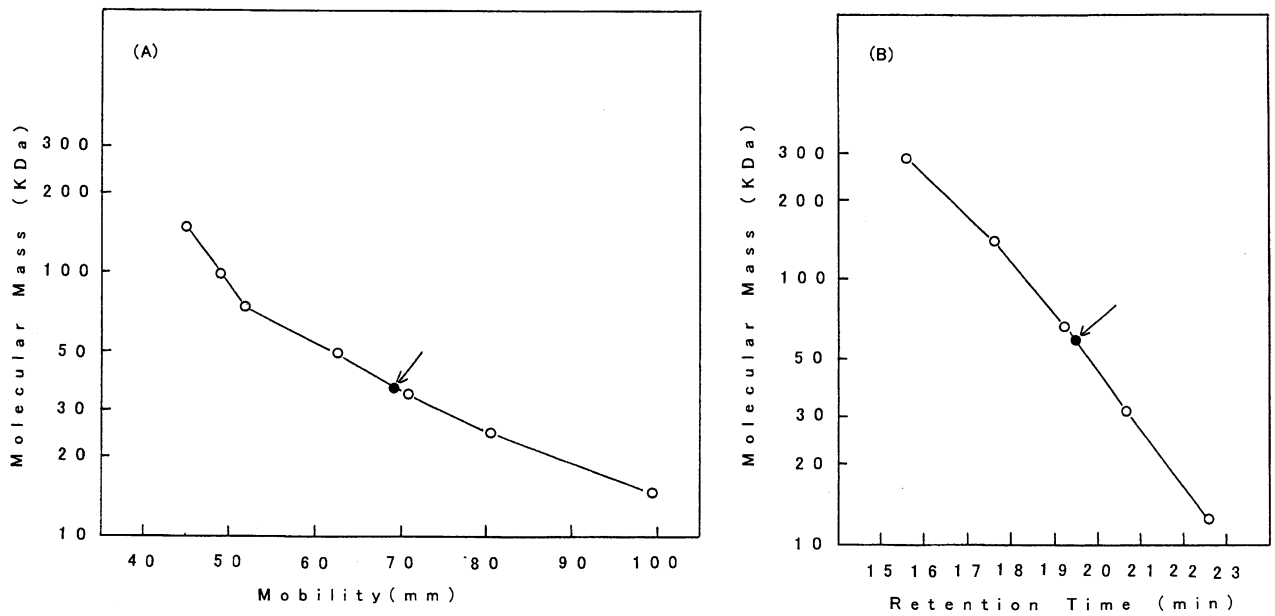

Fig. 3. (A) Estimation of the molecular mass by SDS-PAGE. Standard marker proteins, Takara perfect protein markers (150,000; 100,000; 75,000; 50,000; 35,000; 25,000; 15,000). (B) Estimation of the molecular mass by gel filtration. Standard marker proteins, glutamate dehydrogenase (290,000); lactate dehydrogenase (142,000); enolase (67,000); myokinase (32,000); cytochrome c (12,400).

3.2. Effect of pH and temperature on enzyme activity

The effect of pH on the enzyme activity was investigated in the pH range 4.0–9.0 at 45 °C. As shown in Fig. 2A, maximum activity was obtained at pH 5.5. The enzyme reaction was performed in the range 30–70 °C at pH 5.5. As shown in Fig. 2B, maximum activity was obtained at 45 °C.

3.3. Thermal stability

The enzyme solution was heated at various temperatures for 20 min at pH 5.5, after that residual activities were measured at pH 5.5 and 45 °C. As shown in Fig. 2C, the enzyme was stable up to 75 °C, but it was quickly inactivated at 80 °C. This heat stability was highest in the inulin fructotransferases (DFA I-producing) reported until now (Kushibe et al., 1993; Seki et al., 1989; Ueda et al., 1994). For the industrial application of the enzyme, heat stability is an important factor. Therefore, it will be advantageous to use this enzyme for the production of DFA I.

3.4. Molecular mass estimation

Fig. 3A 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 37,000. Fig. 3B shows the result of molecular mass estimation by gel filtration with TSK-gel G3000SWXL. The molecular mass was estimated to be 60,000. From these results, the enzyme was considered to be a dimmer. On the inulin fructotransferases (DFA I-producing) from *Arthrobacter* species, two enzymes were reported (Seki et al., 1989; Ueda et al., 1994). These enzymes were the monomers. The enzyme from *A. pascens* a62-1 was a dimer, and was different from these enzymes. Table 2 summarizes the comparison of some properties of inulin fructotransferase (DFA I-producing) from different microorganisms.

Table 2
Comparison of properties of inulin fructotransferaseases (DFA I-producing) from different microorganisms

Microorganisms	Optimum		Heat stability	Molecular mass (kDa)		Reference
	pH	°C	°C	SDS-PAGE	Gel filtration	
A. pascens a62-1	5.5	45	75	37	60	This work
A. globiformis S14-3	6.0	40	70	39	46	Seki et al. (1989)
Arthrobacter sp. MCI-2493	6.0	50	70	40	40	Ueda et al. (1994)
Streptomyces sp. MCI-2524	6.0	55	65	36	70	Kushibe et al. (1993)

Table 3
^{13}C NMR chemical shifts of main product and standard DFA I

Assignment carbon atom number	Chemical shifts of ^{13}C NMR of			
	Main product		Standard DFA I[a]	
	(α)	(β)	(α)	(β)
1	65.4	63.9	64.5	63.1
2	105.2	101.5	104.4	100.7
3	84.6	86.2	83.7	85.4
4	79.6	77.2	78.8	76.4
5	83.9	80.4	83.1	79.6
6	64.4	65.2	63.6	64.4

[a] Reference: Haraguchi et al. (1995).

3.5. N-terminal amino acid sequence

The N-terminal amino acid sequence (20 amino acid residues) was determined as Ala-Asn-Thr-Val-Tyr-Asp-Val-Thr-Thr-Trp-Ser-Gly-Ala-Thr-Ile-Ser-Pro-Tyr-Val-Asp-. On the N-terminal amino acid sequence of inulin fructotransferase (DFA I-producing), only the sequence of the enzyme from *A. globiformis* S14-3 was reported (Seki et al., 1989). The amino acid sequence (20 amino acid residues) of N-terminal region of the enzyme from *A. pascens* a62-1 was same as that of the enzyme from *A. globiformis* S14-3. But, as shown in Table 2, the properties of the enzyme from *A. pascens* a62-1 and *A. globiformis* S14-3 were different. Therefore, it was estimated that the differences arose from the difference of the amino acid sequences of internal to C-terminal region of the two enzymes.

3.6. Reaction products

The reaction mixture, after the exhaustive enzyme reaction, was analyzed by paper chromatography as mentioned in Section 2. The Rf value for the main reaction product and two residual oligosaccharides (minor products) were 0.88, 0.48, and 0.40, respectively. The Rf values for the standard materials (DFA I, GF_2 (1-kestose), GF_3 (nystose), GF_4 (fructofuranosyl nystose) were 0.90, 0.54, 0.48, and 0.39, respectively (data not shown). Therefore, the residual oligosaccharides (minor products) were estimated to be GF_3, and GF_4. The main reaction product was prepared from inulin by the method described in Section 2. The ^{13}C NMR spectrum of the main reaction product was recorded. As shown in Table 3, ^{13}C NMR data of the main reaction product agreed well with those of standard DFA I (Haraguchi et al., 1995).

4. Conclusion

The inulin fructotransferase (DFA I-producing) from *A. pascens* a62-1 was purified and its properties were investigated. The enzyme showed maximum activity at pH 5.5 and 45 °C. The enzyme activity was stable up to 75 °C, and this thermal stability was highest in the enzymes reported until now. The molecular mass of the enzyme was estimated to be 37,000 by SDS-PAGE and 60,000 by gel filtration. Therefore, the enzyme was considered to be a dimer.

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