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Purification and properties of a heat stable inulin fructotransferase (DFA III-producing) from *Arthrobacter pascens* T13-2

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Abstract

An inulin fructotransferase (DFA III-producing) [EC 2.4.1.93] from *Arthrobacter pascens* T13-2 was purified and the properties of the enzyme were investigated. The enzyme was purified from a culture supernatant of the microorganism 18.5-fold with a yield of 13.1% by Super Q Toyopearl chromatographies and a butyl Toyopearl chromatography. It gave a single band on SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The molecular mass of the enzyme was estimated to be 44 000 by SDS-PAGE and 79 000 by gel filtration and was therefore considered to be a dimer. The N-terminal amino acid sequence was determined as Ala-Gln-Asp-Ala-Lys-Ala-Gly-Pro-Phe-Asn-Ser-Pro-Asn-Thr-Tyr-Asp-Val-Thr. The enzyme showed maximum activity at pH 5.5–6.0. The optimum temperature for the enzyme activity was 50 °C. The enzyme was stable up to 75 °C. The enzyme activity was inhibited strongly by Hg^{2+}, and inhibited slightly by Fe^{3+}, and Zn^{2+}. An immobilized enzyme was prepared using Chitopearl BCW3510 as a carrier. The immobilized enzyme was able to use eight times without a significant loss of the enzyme activity.

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

1. Introduction

Inulin is a polysaccharide contained in chicory, dahlia, Jerusalem artichoke and other plants. Its chemical structure is a beta-2,1 linked fructose polymer terminated with a sucrose residue.

Studies of inulin decomposing enzymes, inulinases [EC 3.2.1.7] from yeasts and molds were reported in the past. A new type of inulin decomposing enzyme produced by *Arthrobacter ureafaciens* was discovered (Uchiyama, Niwa, & Tanaka, 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]. Subsequently, there have been several reports on the inulin fructotransferase (DFA III-producing) from *Arthrobacter* species (Haraguchi et al., 1988; Kawamura, Takahashi, & Uchiyama, 1988; Yokota, Enomoto, & Tomita, 1991). Kang, Kim, Chang, and Kim (1998) reported on the enzyme from *Bacillus* sp.

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

Recently, we isolated a microorganism, strain T13-2, which produced a heat stable inulin fructotransferase (DFA III-producing) in the culture broth. Through taxonomical studies, this strain was identified as *Arthrobacter pascens* T13-2. In this paper, we describe on the purification and properties of inulin fructotransferase (DFA III-producing) from *A. pascens* T13-2, and for the first time, we report the immobilization of the enzyme and its repeated use.

2. Materials and methods

2.1. Cultivation of microorganism

For a pre-culture, the microorganism was cultured in a 500 ml shaking flask at 30 °C for 24 h which containing a medium (100 ml). 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

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Table 1
Purification of the inulin fructotransferase (DFA III-producing) from *A. pascens* T13-2 (the purification was started from 1960 ml of the crude enzyme)

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Crude enzyme	123	1600	13.0	100
Super Q-Toyopearl (1st)	6.07	723	119	45.2
Butyl-Toyopearl	1.52	332	218	20.8
Super Q-Toyopearl (2nd)	0.870	209	240	13.1

the cultivation, the cells were removed by centrifugation ($8000 \times g$, 30 min) and the supernatant was used as the crude enzyme solution.

2.2. Standard assay methods

For the measurement of the enzyme activity, 0.1 M sodium phosphate buffer, pH 6.0 (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 III produced was determined by HPLC (column, Shim-pack CLC ODS, 4.6 mm × 25 cm (Shimadzu Co. Ltd, Kyoto); mobile phase, water; flow rate, 0.6 ml/min; detector, ERC 7510 RI detector (Erma optical works, Japan)). One unit of the enzyme was defined as which can produce 1 μmol of DFA III per min at pH 6.0 and 50 °C. Protein concentrations were determined with the method of Lowry, Rosebrough, Farr, and Randall (1951) using bovine serum albumin as a standard.

2.3. Purification of the enzyme

The crude enzyme solution was dialyzed against, 10 mM Tris–HCl buffer, pH 8.5. The dialysate was applied on a column of Super Q Toypearl 650 M (1st 2.5 cm × 17 cm). The elution was performed with linear 0 to 0.6 M NaCl gradient in the same buffer. The fractions showing the enzyme activity were pooled and dialyzed against 10 mM Tris–HCl buffer, pH 8.5, containing 100 g/l of ammonium sulfate. This dialysate was applied on a column of butyl Toyopearl 650 M (1.5 cm × 12 cm) equilibrated with 10 mM Tris–HCl buffer, pH 8.5, containing 100 g/l of ammonium sulfate. The elution was performed with linear 100 to 0 g/l ammonium sulfate gradient in the same buffer. The fractions containing the enzyme activity were pooled and dialyzed against 10 mM Tris–HCl buffer, pH 8.5. The dialysate was applied to a column of Super Q Toyopearl 650 M (2nd, 1.5 cm × 14 cm). The elution was performed with linear 0 to 0.5 M NaCl gradient in the same buffer. The fractions showing the enzyme activity were pooled and 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 (HPLC column; TSK-gel G3000SWXL, Tohsoh Co. Ltd, Japan, mobile phase; 50 mM sodium phosphate buffer, pH 7.0, containing 0.3 M NaCl; flow rate, 1.0 ml/min; detection, UV280 nm).

2.5. Amino acid sequencing

The purified enzyme was electro-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 peptide sequencer (Hewlett Packard Co. Ltd, USA).

2.6. Preparation of reaction product

For the preparation of the reaction products after the exhaustive enzyme reaction, 0.1 M, sodium phosphate buffer pH 6.0 (0.5 ml), the purified enzyme solution (0.5 ml, 7 units/ml) and 5% inulin (1.0 ml) were mixed. The enzyme reaction was performed at 50 °C for 4 h, and the enzyme reaction was stopped by heating at 100 °C for 7 min. After cooling, the reaction products were analyzed by HPLC (column, Zorbax ODS (4.6 mm × 25 cm), Shimadzu Co. Ltd, Kyoto; mobile phase, 0.2% acetonitrile; flow rate 0.6 ml/min).

2.7. Preparation of an immobilized enzyme

For the preparation of an immobilized enzyme, Chitopearl

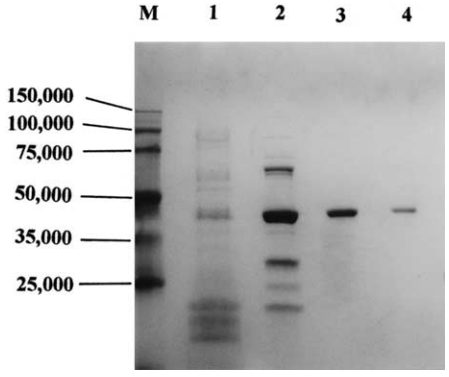

Fig. 1. SDS-PAGE of the intermediate fractions and the purified enzyme. Lane M, molecular mass standard markers; lane 1, crude enzyme; lane 2, Super Q Toyopearl (1st) fraction; lane 3, butyl Toyopearl fraction; lane 4, Super Q Toyopearl (2nd) fraction (purified enzyme).

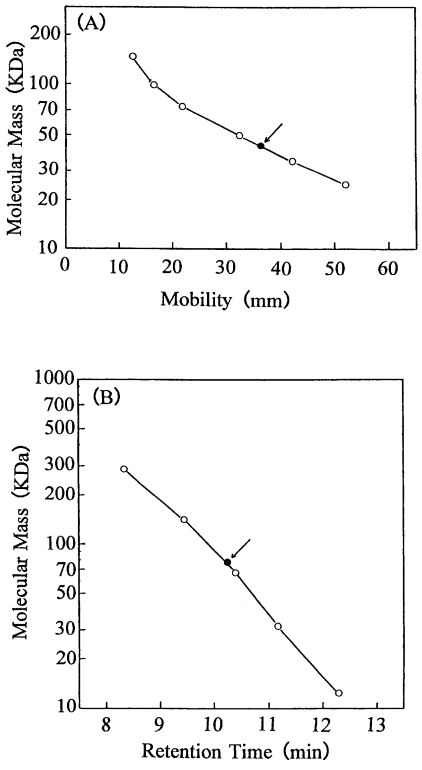

Fig. 2. (A) Estimation of the molecular mass by SDS-PAGE. Standard marker proteins, Takara perfect protein markers (150 000; 100 000; 75 000; 50 000; 25 000). (B) Estimation of the molecular mass by gel filtration. Standard marker proteins, glutamate dehydrogenase (290 000); lactate dehydrogenase (142 000); enolase (67 000); myokinase (32 000); cytochrome c (12 400).

BCW3510 (5 g, Fuji Boseki, Co. Ltd, Japan) was suspended in 10 mM sodium phosphate buffer, pH 6.0 (100 ml). The purified enzyme solution (10 ml, 7 units/ml) was added to it and incubated at 30 °C for 1 h with shaking. After that, the prepared immobilized enzyme was washed once with water. The washed enzyme was treated with 2.5% glutaraldehyde for 1 h at 30 °C to stabilize the immobilized enzyme. The treated immobilized enzyme was washed thoroughly with water, and used as the prepared immobilized enzyme.

2.8. The enzyme reaction using the immobilized enzyme

The immobilized enzyme (3 g) was put in a 50 ml Erlenmeyer flask, and the reaction was started with the addition of 1% inulin (5.0 ml) containing 10 mM sodium phosphate buffer, pH 6.0. The enzyme reaction was performed at 50 °C for 50 min with shaking. After the reaction, the reaction mixture was moved to a test tube and heated at 100 °C for 7 min to stop the enzyme reaction. After cooling, the DFA III produced was determined by HPLC (column: Shim-pack CLC ODS, mobile phase: water; flow rate, 0.6 ml/min). To the Erlenmeyer flask containing the immobilized enzyme, the new inulin solution was added and the enzyme reaction was performed repeatedly.

3. Results and discussion

3.1. Purification of the enzyme

Table 1 shows the summary of the enzyme purification. The enzyme was purified 18.5-fold with a yield of 13.1% by the two times of DEAE-Toyopearl chromatography and a butyl Toyoperal chromatography. The purified enzyme was analyzed by SDS-PAGE. As shown in Fig. 1, it gave a single band on SDS-PAGE.

3.2. Molecular mass estimation

Fig. 2(A) shows plots of logarithmic molecular mass vs. protein mobility on SDS-PAGE. The molecular mass of the enzyme was estimated to be 44 000. Fig. 2(B) shows the result of molecular mass estimation by gel filtration with TSK-gel G3000SWXL. The molecular mass was estimated to be 79 000. From these results, the enzyme was considered to be a dimer.

3.3. N-terminal amino acid sequence

The N-terminal 18 amino acids were determined as Ala-Gln-Asp-Ala-Lys-Ala-Gly-Pro-Phe-Asn-Ser-Pro-Asn-Thr-Tyr-Asp-Val-Thr-. Fig. 3 shows the comparison of N-terminal amino acid sequence of inulin fructotransferases (DFA III-producing) from various microorganisms (Haraguchi, Mori, & Hayashi, 2000; Kang et al., 1998; Sakurai, Yokota, & Tomita, 1997). At the N-terminal seven amino acids, the sequence of the enzyme from *A. pascens* T13-2 has no distinct homology with those of other microorganisms. But the sequence of 10th to 18th amino acid of the enzyme was same as that of the sequence of *Arthrobacter globiformis* C11-1. The N-terminal amino acid sequence of the enzyme from *A. pascens* T13-2 has no distinct homology with that of inulinase [EC 3.2.17] from *Kluyveromyces marxianus* (Laloux, Cassart, Delcour, Beeumen, & Vandenhaute, 1991).

3.4. Effects of pH and temperature on the enzyme activity

The effect of pH on the enzyme activity was investigated in the pH range 4.0–9.0 at 50 °C. As shown in Fig. 4(A), the maximum activity was obtained at pH 5.5–6.0. The enzyme

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C11-1 ADGQQGAPLNSPNTYDVT
H65-7 ADSTEET-----NRYDVT
Snu-7 ADGQDGAPLNQVNTYD--
T13-2 AQDAKAGPFNSPNTYDVT
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Fig. 3. Comparison of N-terminal amino acid sequences of inulin fructotransferase (DFA III-producing) from various microorganisms. C11-1, the N-terminal amino acid sequence of the enzyme from *A. globiformis* C11-1. H65-7, the N-terminal sequence of the enzyme from *Arthrobacter* sp. H65-7. Snu-7, the N-terminal sequence of the enzyme from *Bacillus* sp. Snu-7. T13-2, the N-terminal sequence of the enzyme from *A. pascens* T13-2. The identical residues in the sequence are presented by white letters in black boxes.

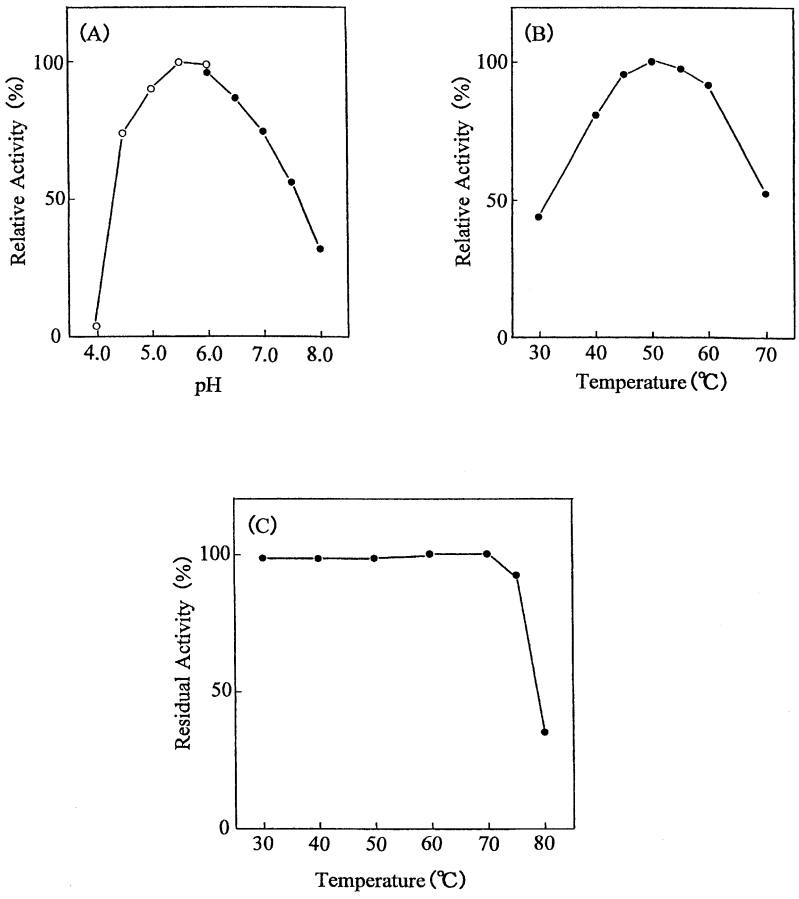

Fig. 4. (A) Effect of pH on the enzyme activity. (○), citrate buffer; (●), phosphate buffer. (B) Effect of temperature on the enzyme activity. (C) Thermal stability of the enzyme.

reaction was performed in the temperature range 30–70 °C at pH 6.0. As shown in Fig. 4(B), the maximum activity was observed at 50 °C.

3.5. Thermal stability

The enzyme solution was heated at various temperatures for 20 min at pH 6.0. After that the enzyme reaction was performed at 50 °C and pH 6.0. As shown in Fig. 4(C), it was stable up to 75 °C, but it was quickly inactivated at 80 °C. 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 III. Table 2 summarizes the comparison of some properties of inulin fructotransferase (DFA III-producing) from different microorganisms.

3.6. Effects of metal ions and other chemicals

The enzyme reaction was performed in the reaction mixture containing 1 mM of each metal ions or reagents at pH 6.0 and 50 °C. As shown in Table 3, the enzyme activity was inhibited strongly by Hg^{2+}, and was inhibited slightly by Fe^{3+} and Zn^{2+}.

Table 2
Comparison of properties of inulin fructotransferases (DFA III-producing) from different microorganisms

Microorganisms	Optimum		Heat stability ((C)	Molecular mass (kDa)		Reference
	pH	(C		SDS-PAGE	Gel filtration	
A. pascens T13-2	5.5–6.0	50	75	44	79	This study
A. ureafaciens	6.0	50	50		80	Uchiyama et al. (1973)
A. globiformis C11-1	5.0	55	75	45	50	Haraguchi et al. (1988)
A. ilicis OKU17B	5.5	60	70	27	50	Kawamura et al. (1988)
Arthrobacter sp H65-7	5.5	60	70	49	100	Yokota et al. (1991)
Bacillus sp. snu-7	6.0	40	60	62		Kang et al. (1998)

Table 3
Effects of metal ions and other chemicals on the enzyme activity (the metals were used as the chloride salts)

Materials (1 mM)	Remaining activity (%)
None	100
Ba^{2+}	100
Ca^{2+}	101
Co^{2+}	99
Cu^{2+}	94
Fe^{3+}	68
Hg^{2+}	0
Mg^{2+}	99
Ni^{2+}	100
Zn^{2+}	74
EDTA-2Na	102
SDS	101

3.7. Reaction products

The reaction mixture after the exhaustive enzyme reaction, was analyzed by HPLC as mentioned in Section 2. The retention times for the main products (DFA III) and two residual oligosaccharides were 3.3 min (main product), 4.5 and 7.4 min, respectively. The retention time for the standard materials (DFA III, GF2 (1-kestose), GF3 (nystose), and GF4 (fructofuranosyl nystose)) were 3.3, 3.5, 4.5 and 7.3 min, respectively (data not shown). Therefore, the residual oligosaccharides (minor products) of the enzyme reaction were estimated to be GF3 and GF4.

3.8. Reaction with the immobilized enzyme

The prepared immobilized enzyme has an activity of 7.0 units/g (wet weight). Therefore, the recovery of the enzyme activity on immobilization was about 50%. Fig. 5 shows the result of repeated use of the immobilized enzyme. The reaction was performed eight times and there was no significant loss of enzyme activity during the repetitive use of the immobilized enzyme. This result suggests that a bioreactor using the immobilized enzyme is applicable for the industrial production of the oligosaccharide DFA III.

4. Conclusions

The inulin fructotransferase (DFA III-producing) from *A. pascens* T13-2 was purified and its properties were investigated. The molecular mass of the enzyme was estimated to be 44 000 by SDS-PAGE and 79 000 by gel filtration. Therefore, the enzyme was considered to be a dimer. The enzyme showed maximum activity at pH 6.0 and 50 °C. The enzyme activity was stable up to 75 °C. Using Chitopearl BCW 3510 as a carrier, the immobilized enzyme was readily prepared. The immobilized enzyme could be used repeatedly without a significant loss of the activity.

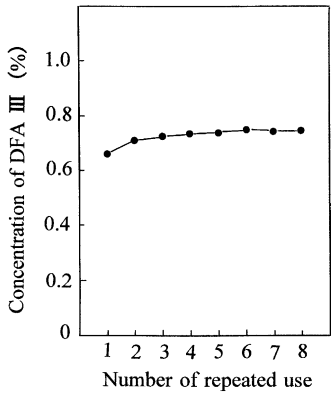

Fig. 5. Repeated use of the immobilized enzyme.

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