Available online at www.sciencedirect.com
SCIENCE DIRECT
Carbohydrate Polymers 59 (2005) 411–416
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Thermostable inulin fructotransferase (DFA III-producing) from *Arthrobacter* sp. L68-1

Kazutomo Haraguchi*, Mitsuru Yoshida, Ken'ichi Ohtsubo

National Food Research Institute, 2-1-12 Kannondai, Tsukuba-shi, Ibaraki 305-8642, Japan

Received 9 September 2003; revised 17 May 2004; accepted 16 June 2004
Available online 2 December 2004

Abstract

An inulin fructotransferase (DFA III-producing) [EC 2.4.1.93] from Arthrobacter sp. L68-1 was purified and characterised. The enzyme was purified from culture supernatant of the microorganism 54.2-fold with a yield of 16.0% using DEAE-Toyopearl chromatography (repeated twice) and Super Q-Toyopearl chromatography. The enzyme showed maximum activity at pH 5.5–6.0 and 55 °C. The enzyme activity was stable up to 80 °C after 1 h heat treatment. This heat stability was the highest of the inulin fructotransferases (DFA III-producing) reported to date. The molecular mass of the enzyme was estimated to be 43 kDa by SDS-PAGE and 73 kDa by gel filtration, and was considered to be a dimer. The N-terminal amino acid sequence (18 amino acid residues) was determined as AEETKGGPFNSPNAYDVT.

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

1. Introduction

Inulin is a storage polysaccharide contained in chicory, dahlia, Jerusalem artichoke and other plants. The chemical structure of inulin is a β-2, 1 linked fructose polymer terminated with a sucrose residue. In studies of inulin decomposing enzymes, inulinase [EC 3.2.1.7] from *Penicillum* sp. was reported (Nakamura & Hoashi, 1969). Afterwards, 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, 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.

Chicory is a leading candidate alternative crop to sugar beet. The DFA III, which can produce from chicory, has half the sweetness of sucrose. Recently, Goto (2002) reported that the DFA III did not cause an increase of blood sugar after assimilation (using rat). Therefore, the DFA III has potential as a new type of a low calorie sweetener.

Recently, we isolated a microorganism, strain L68-1, which produced a thermostable inulin fructotransferase (DFA III-producing) in the culture supernatant. Through taxonomical studies, the microorganism was identified as *Arthrobacter* sp. L68-1. In this paper, we describe the purification and properties of the enzyme.

2. Materials and methods

2.1. Microorganism and its cultivation

We isolated a microorganism from a soil sample collected in the Ibaraki prefecture. The taxonomical study was performed by NCIMB Japan, and the microorganism was identified as *Arthrobacter* sp. L68-1. For a pre-culture,

* Corresponding author. Fax: +81 029 838 7996.
E-mail address: haraguti@nfri.affrc.go.jp (K. Haraguchi).

0144-8617/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2004.06.046

Table 1
Purification of inulin fructotransferase (DFA III-producing) from *Arthrobacter* sp. L68-1

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Recovery (%)
Crude enzyme	511	8770	17.2	1	100
DEAE-Toyopearl (first)	33.5	6300	188	10.9	71.8
DEAE-Toyopearl (second)	11.8	3960	336	19.5	45.2
Super Q Toyopearl	1.50	1400	933	54.2	16.0

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 another medium and cultured at 30 °C, for 24 h. The medium was composed of 0.4% $Na_2HPO_4 \cdot 12H_2O$, 0.1% KH_2PO4, 0.1% $NaNO_3$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $CaCl_2 \cdot 2H_2O$, 0.001% $CaCl_2 \cdot 2H_2O$, 0.1% Tryptone (Difco), and 0.5% inulin, pH 7.0. After 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 55 °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; detector, refractive index detector). One unit of the enzyme was defined as the amount of enzyme which can produce 1 μmol of DFA III per min at pH 5.5 and 55 °C. Protein concentrations were determined by 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 dialyzed enzyme solution was applied on a column of DEAE-Toyopearl 650M (2.5 cm × 17 cm, Tohsoh Co. Ltd, Japan) 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 Tris–HCl buffer, pH 8.5. The dialyzed enzyme solution was applied on a column of DEAE-Toyopearl 650M (1.5 cm × 12 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 5 mM sodium phosphate buffer, pH 8.0. The dialyzed enzyme solution was applied on a column of Super Q-Toyoperal equilibrated with 5 mM sodium phosphate buffer pH 8.0. 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 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 7.0, containing 0.5 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). And the amino acid sequence of N-terminal region of the enzyme was analyzed by automated Edman degradation with a G1005A protein sequencer (Hewlett Packard Co. Ltd, USA).

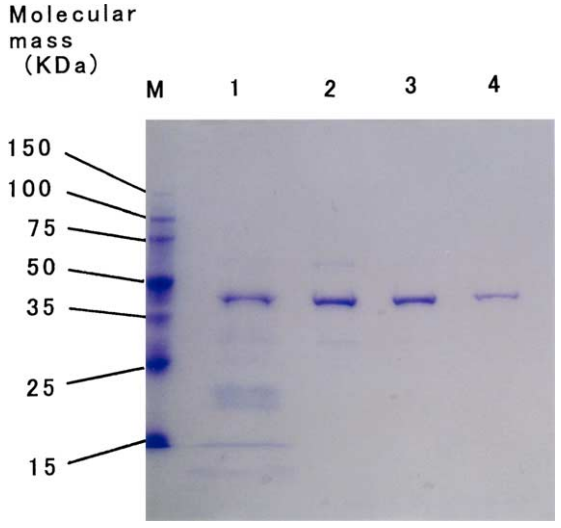

Fig. 1. SDS-PAGE of the intermediate fractions and the purified enzyme. Lane M, Molecular mass standard markers; lane 1, Crude enzyme; lane 2, DEAE-Toyopearl (first) fraction; lane 3, DEAE-Toyopearl (second) fraction; lane 4, Super Q-Toypearl fraction (purified enzyme).

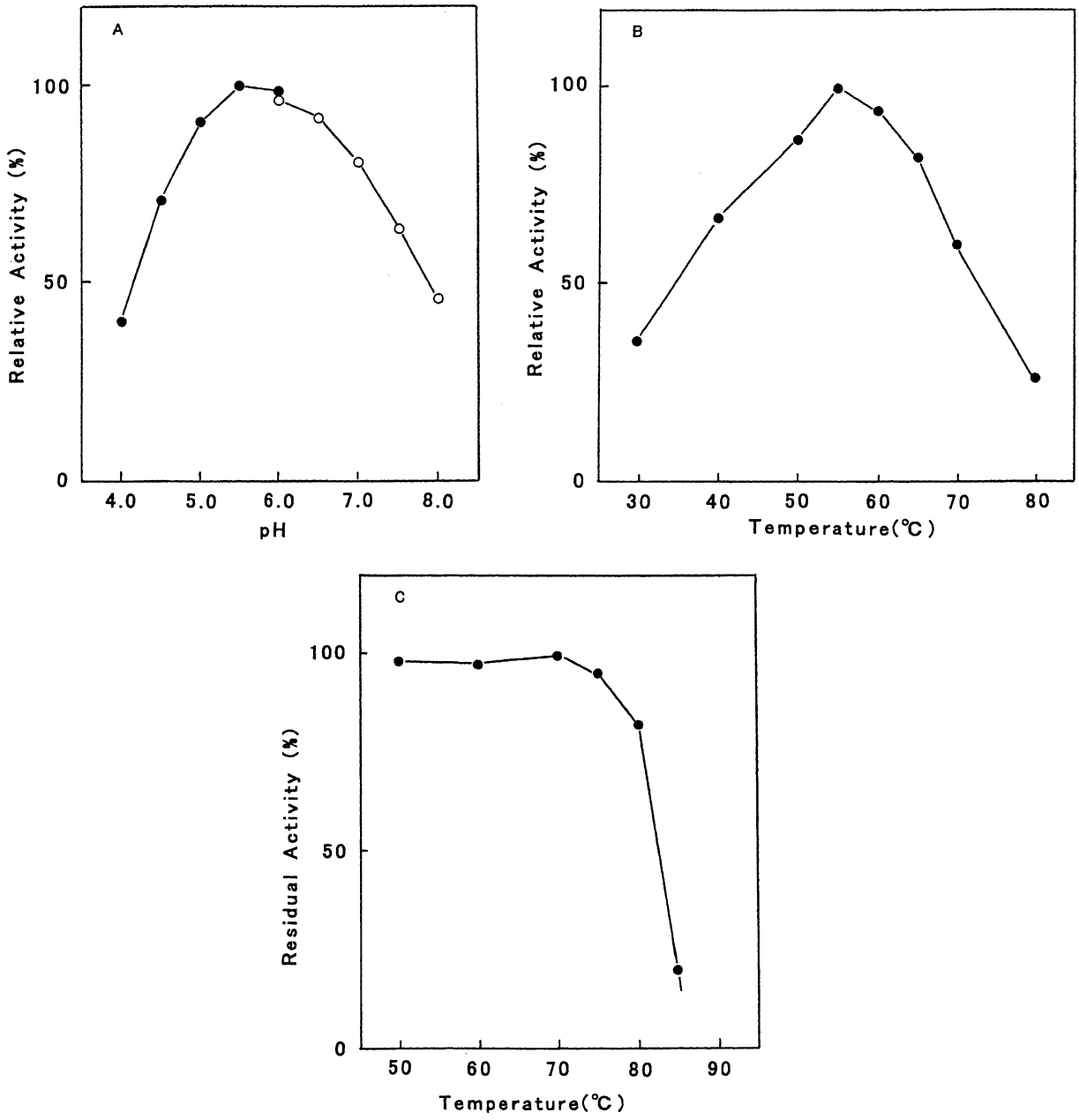

Fig. 2. (A) Effect of pH on the enzyme activity. (●) Citrate buffer; (○) Phosphate buffer; (B) effect of temperature on the enzyme activity; (C) Thermal stability of the enzyme.

2.6. Preparation of reaction products

For the preparation of the reaction products, 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 55 °C, for 20 h and 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 (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. 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 applied to 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 product were pooled (The purity was checked by HPLC). The fraction was concentrated by freeze-drying and used as a sample of

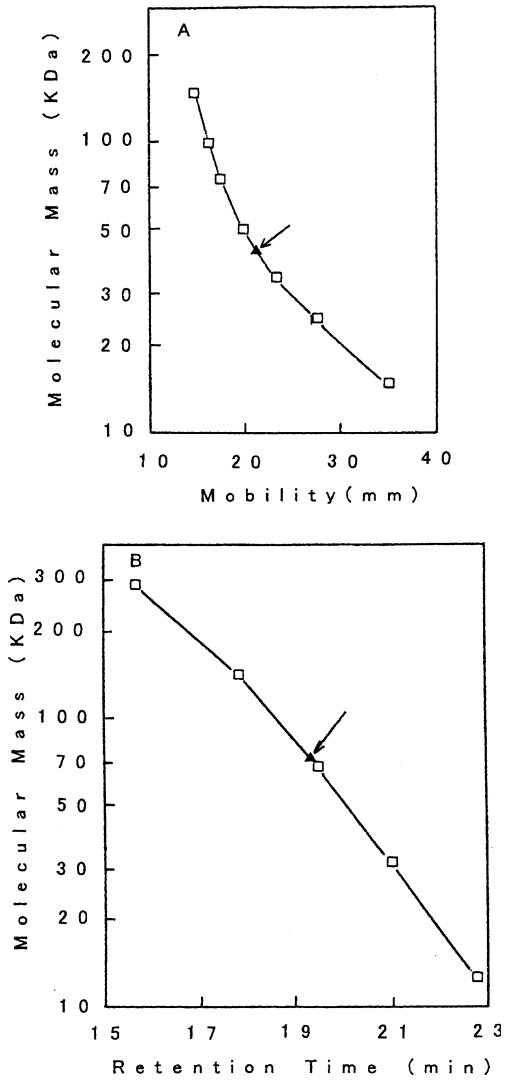

Fig. 3. (A) Estimation of the molecular mass by SDS-PAGE. Standard marker proteins, Takara perfect protein markers (150; 100; 75; 50; 35; 25; 15 kDa). (B) Estimation of the molecular mass by gel filtration. Standard marker proteins, glutamate dehydrogenase (290 kDa); lactate dehydrogenase (142 kDa); enolase (67 kDa); myokinase (32 kDa); cytochrome *c* (12.4 kDa).

C-13 NMR. The C-13 NMR spectrum was recorded in D_2O with a BRX 600 spectrometer (Bruker Co. Ltd, Germany) using 3-trimethylsilyl-1-propanesulfonic acid sodium salt (DSS) as a standard material.

3. Results and discussion

3.1. Purification of enzyme

Table 1 shows a summary of the enzyme purification. The enzyme was purified 54.2-fold with a yield of 16.0% by two times of DEAE-Toyopearl chromatography and a Super Q Toyopearl chromatography. The purified enzyme was analyzed by SDS-PAGE, and as shown in Fig. 1, it gave a single band.

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 55 °C. As shown in Fig. 2(A), maximum activity was obtained at pH 5.5–6.0. The enzyme reaction was performed in the range 30–80 °C at pH 5.5. As shown in Fig. 2(B), maximum activity was obtained at 55 °C. For the industrial application of the enzyme, it is desirable to react at temperature above 60 °C of at least avoid microbial problems. The enzyme retains more than 90% of maximum activity at 60 °C (Fig. 2(B)). This property makes it suitable for industrial applications.

3.3. Thermal stability

The enzyme solution was heated at various temperatures for 1 h at pH 5.5, after that the residual activities were measured at pH 5.5 and 55 °C. As shown in Fig. 2(C), the enzyme was stable up to 80 °C, but it was inactivated at 85 °C. This heat stability was highest in the inulin fructotransferases (DFA III-producing) reported to date. For the industrial application of the enzyme, heat stability is one of the most important factors. Therefore, it will be advantageous to use this enzyme for the large scale production of DFA III.

3.4. Molecular mass estimation

Fig. 3(A) 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 43 kDa. Fig. 3(B) shows the result of molecular mass

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

Microorganisms	Optimum		Heat stability °C (time)	Molecular mass (kDa)		K_m (mM)	References
	pH	°C		SDS-PAGE	Gel filtration		
Arthrobacter sp. L68-1	5.5–6.0	55	80 (60 min)	43	73	10	This work
A. ureafaciens	6.0	50	50 (30 min)		80		Uchiyama et al. (1973)
A. globiformis C11-1	5.0	55	75 (20 min)	45	50		Haraguchi et al. (1988)
A. ilicis OKU17B	5.5	60	70 (30 min)	27	50		Kawamura et al. (1988)
Arthrobacter sp. H65-7	5.5	60	70 (20 min)	49	100	0.8	Yokota, Enomoto, & Tomita (1991)
A. pascens T13-2	5.5–6.0	50	75 (20 min)	44	79		Haraguchi et al. (2002)
Bacillus sp. Snu-7	6.0	40	60 (10 min)	62		5.4	Kang et al. (1998)

Fig. 4. Comparison of N-terminal amino acid sequence of inulin fructotransferase (DFA III-producing) from various microorganisms. L68-1, The sequence of *Arthrobacter* sp. L68-1; C11-1, The sequence of *A. globiformis* C11-1; H65-7, The sequence of *Arthrobacter* sp. H65-7; T13-2 The sequence of *A. pascens* T13-2; Snu-7, The sequence of *Bacillus* sp. Snu-7. The identical residues are presented by white letters in black boxes.

estimation by gel filtration with TSK-gel G3000SWXL. The molecular mass was estimated to be 73 kDa. From these results, the enzyme was considered to be a dimer. Table 2 summarizes the comparison of some properties of inulin fructotransferase (DFA III-producing) from different microorganisms.

3.5. N-terminal amino acid sequence

N-terminal amino acid sequence was determined as AEETKGGPFNSPNAYDVT. Fig. 4 shows the comparison of N-terminal amino acid sequence of inulin fructotransferase (DFA III-producing) from various microorganisms. At the N-terminal six amino acid residues, the sequence of *Arthrobacter* sp. L68-1 has no distinct homology with those of other microorganisms. But the sequence of 7th–18th amino acid residues of the enzyme was same as that of *Arthorbacter pascens* T13-2 (Haraguchi et al., 2002) except the 14th amino acid residue.

3.6. Estimation of K_m value

The enzyme reaction was performed at pH 5.5 and 55 °C at various concentrations of inulin (molecular mass assumed as 5000 Da). The double-reciprocal plots of the reaction rate against the substrate concentrations were performed. As shown in Fig. 5, the K_m value at the conditions was estimated to be 10 mM. This value was highest among the K_m value reported to date (Table 2).

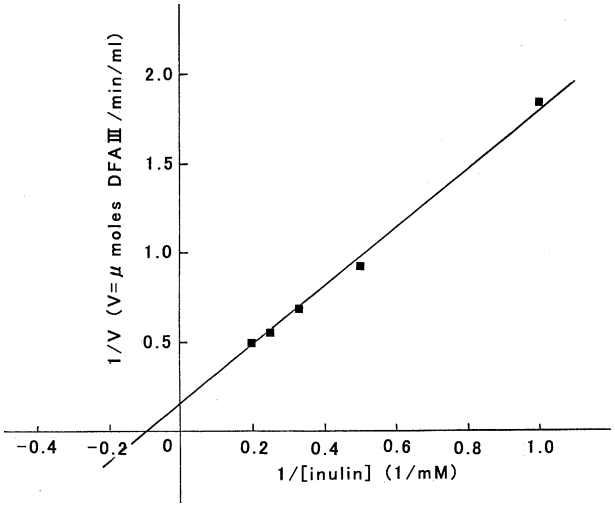

Fig. 5. Estimation of K_m value of the enzyme by double reciprocal plots.

Table 3
C-13 NMR chemical shifts of main reaction product and DFA III

Chemical shifts of C-13 NMR of				
Assignment carbon atom number	Main reaction product		Standard DFA III	
	(α)	(β)	(α)	(β)
1	66.1	61.6	66.2	61.7
2	106.3	104.0	106.3	104.2
3	83.8	84.5	83.9	84.5
4	78.0	74.9	78.1	75.0
5	82.8	81.7	82.9	81.8
6	63.3	63.8	63.3	63.8

References: Yokota, Hirayama, et al. (1991).

3.7. Reaction products

The reaction mixture, after the exhaustive enzyme reaction, was analyzed by paper chromatography as mentioned in materials and methods. The R_f value for the main reaction product and two residual oligosaccharides (minor products) were 0.98, 0.53, and 0.41, respectively. The R_f values for the standard materials (DFA III, GF_2 (1-kestose), GF_3 (nystose), GF_4 (fructofuranosyl nystose) were 0.98, 0.61, 0.52, and 0.41, 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 materials and methods.

The C-13 NMR spectrum of the main reaction product was recorded. As shown in Table 3, C-13 NMR data of the main reaction product agreed well with those of standard DFA III (Yokota, Hirayama, et al., 1991).

3.8. Substrate specificity

The enzyme reaction was carried out at 55 °C for 5 h in a reaction mixture containing 25 mM citrate buffer, pH 5.5, 0.5 ml of the purified enzyme (22 units) and the following compounds as a substrate instead of inulin; sucrose, 1-kestose (GF_2), nystose (GF_3), fructofuranosyl nystose (GF_4), and raffinose at a concentration of 1%. From the results of paper chromatography analysis of the reaction products, it was found that none of these oligosaccharides was a substrate of the enzyme (data not shown).

4. Conclusion

The thermostable inulin fructotransferase (DFA III-producing) from *Arthrobacter* sp. L68-1 was purified and its properties were investigated. The enzyme showed

maximum activity at pH 5.5–6.0 and 55 °C. The enzyme activity was stable up to 80 °C, and this thermal stability was highest in the enzymes reported to date. The molecular mass of the enzyme was estimated to be 43 kDa by SDS-PAGE and 73 kDa by gel filtration. Therefore, the enzyme was considered to be a dimer. The N-terminal amino acid sequence was analyzed as AEETKGGPFNSPNAYDVT.

References

Goto, K. (2002). Fructo-oligosaccharides from yacon (in Japanese). *The abstracts of "Bio-Renaissance Program", ministry of agriculture, forestry and fisheries, Japan* (pp. 9–10).

Haraguchi, K., Kishimoto, M., Seki, K., Kobayashi, S., & Kainuma, K. (1988). Purification and properties of inulin fructotransferase (depolymerizing) from *Arthrobacter globiformis*. *Agricultural and Biological Chemistry*, *52*, 291–292.

Haraguchi, K., Yamanaka, T., & Ohtsubo, K. (2002). Purification and properties of a heat stable inulin fructotransferase (DFA III-producing) from *Arthrobacter pascens* T13-2. *Carbohydrate Polymers*, *52*, 117–121.

Kang, S., Kim, W., Chang, Y., & Kim, S. (1998). Purification and properties of inulin fructotransferase (DFA III-producing) from *Bacillus* sp. snu-7. *Bioscience Biotechnology and Biochemistry*, *62*, 628–631.

Kawamura, M., Takahashi, S., & Uchiyama, T. (1988). Purification and some properties of inulin fructotransferase (depolymerizing) from *Arthrobacter ilicis*. *Agricultural and Biological Chemistry*, *52*, 3209–3210.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, *193*, 265–275.

Nakamura, T., & Hoashi, S. (1969). Culture conditions for inulase production by *Penicillum*. *Nippon Nogeikagaku Kaishi*, *43*, 599–605.

Uchiyama, T., Niwa, S., & Tanaka, K. (1973). Purification and properties of *Arthrobacter ureafaciens* inulase II. *Biochimica Biophysica Acta*, *315*, 412–420.

Yokota, A., Hirayama, S., Enomoto, K., Miura, Y., Takao, S., & Tomita, F. (1991). Production of inulin fructotransferase (depolymerizing) by *Arthrobacter* sp. H65-7 and preparation of DFA III from inulin by the enzyme. *Journal of Fermentation and Bioengineering*, *72*, 258–261.

Yokota, A., Enomoto, K., & Tomita, F. (1991). Purification and properties of an inulin fructotransferase (depolymerizing) from *Arthrobacter* sp. H65-7. *Journal of Fermentation and Bioengineering*, *72*, 262–265.