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Inulin fructotransferase (DFAIII-producing) from *Arthrobacter ureafaciens* D13-3

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ABSTRACT

An inulin fructotransferase (DFA III-producing) [EC 2.4.1.93] from *Arthrobacter ureafaciens* D13-3 was purified and characterized. The enzyme was purified from culture supernatant of the microorganism 22.3-fold with a yield of 23.6%. The enzyme showed maximum activity at pH 5.5 and 50 °C. The enzyme activity was stable up to 70 °C after 30 min heat treatment. The molecular mass of the enzyme was estimated to be 40 kDa by SDS-PAGE and 43 kDa by gel filtration, and the enzyme was considered to be a monomer. The smallest fructo-oligosaccharide as the substrate was estimated to be GF_3 (nystose). The N-terminal amino acid sequence (12 amino acid residues) was analyzed as TTVYDTTVDVP.

1. Introduction

Inulin is a 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 molds and yeast were reported in the past. Afterwards, a new type of inulin decomposing enzyme produced by *Arthrobacter ureafaciens* was discovered (Uchiyama, Niwa, & Tanaka, 1973). The enzyme converted inulin into an oligo-saccharide DFA III (di-D-fructofuranose 1,2′:2,3′ dianhydride) and a small amount of other oligo-saccharides. 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; Haraguchi, Yamanaka, & Ohtsubo, 2002; Haraguchi, Yoshida, & Ohtsubo, 2005; Kawamura, Takahashi, & Uchiyama, 1988; Yokota, Enomoto, & Tomita, 1991). Kang, Kim, Chang, and Kim (1998) reported on the enzyme from *Bacillus* sp. We reported on the enzyme from *Leifsonia* sp. (Haraguchi, Yoshida, & Ohtsubo, 2006).

The DFAIII has half the sweetness of sucrose. It was found that the DFA III accelerates the assimilation of minerals (Ca, Fe, and so on) from intestines (Saito & Tomita, 2000). Therefore, the DFAIII has a potential for the improvement of osteoporosis and iron deficiency anemia.

Recently, we isolated a microorganism, strain D13-3, which produced an inulin fructotransferase (DFAIII-producing) in the culture supernatant. Through taxonomic studies, the microorganism was identified as *Arthrobacter ureafaciens* D13-3. Mostly, the minor products of the inulin fructotransferase (DFA III-producing) were GF_3 and GF_4 (Haraguchi et al., 1988, 2002, 2005, 2006; Kawamura et al., 1988; Yokota et al., 1991). Therefore, the smallest substrate for those enzymes was estimated to be GF_5. Though, the smallest substrate for the enzyme of *A. ureafaciens* D13-3 was estimated to be GF_3 (nystose). In this paper, we describe the purification and properties of this type of the enzyme.

2. Materials and methods

2.1. Chemicals

The oligosaccharide DFA III was obtained from Fancl Co. Ltd. (Japan). The standard fructo-oligosaccharides (GF_2, GF_3, GF_4) were obtained from Wako pure chemicals Co. Ltd. (Japan).

2.2. Identification of microorganism

We isolated a microorganism, strain D13-3 (SIID 2513, NCIMB Japan), from a soil sample collected in Ibaraki prefecture, Japan. For the extraction of a genome DNA Prepman method (Applied Biosystems, Co. Ltd., USA) was used. The amplification of 16S rDNA was performed using PCR system 9600 (Applied Biosystems Co. Ltd.). The DNA sequencing was performed with an ABI PRISM 3100 sequencer (Applied Biosystems Co. Ltd.).

2.3. 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. The medium was composed of 0.4% $Na_2HPO_4{\cdot}12H_2O$, 0.1% KH_2PO_4,

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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. The pre-culture was inoculated in a 5 l Erlenmeyer flask containing 1 l of the same medium and cultured at 30 °C, for 24h. After the cultivation, the cells were removed by centrifugation (8000 × g, 30 min) and the supernatant was used as a crude enzyme solution.

2.4. Standard assay methods

For the measurement of the enzyme activity, 0.1 M citrate buffer, pH 5.5 (0.5 ml), the enzyme solution (0.1 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 DFAIII-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 the enzyme which can produce 1 μmole of DFA III per min at pH 5.5 and 50 °C. Protein concentrations were determined with method of Lowry, Rosebrough, Farr, and Randall (1951) using bovine serum albumin as a standard.

2.5. 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 Super Q-Toyopearl 650M (1st, 1.5 cm × 12 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 containing the enzyme activity were pooled and dialyzed against 5 mM phosphate buffer, pH 8.0. The dialyzed solution was applied on a column of Super Q-Toyopearl 650M (2nd, 1.5 cm × 12 cm) equilibrated with 5 mM phosphate buffer, pH 8.0. The elution was performed with linear 0–0.4 M NaCl gradient in the same buffer. The fractions containing the enzyme activity were pooled and used as a purified enzyme solution.

2.6. 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; flow rate, 0.5 ml/min; detection, UV280 nm).

2.7. 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 G1005A protein sequencer (Hewlett Packard Co. Ltd., USA).

Table 1
Taxonomic characteristics of strain D13-3.

Shape and size	Rod: 0.8 × 2.0–3.0 μm (24 h)
Gram staining	Positive
Spore formation	−
Motility	−
Pleomorphism	+
Catalase	+
Oxidase	−
Reduction of nitrate	−
Gelatin hydrolysis	+

2.8. Preparation of reaction products from inulin

For the preparation of the reaction products, 0.1 M citrate buffer, pH5.5 (0.3 ml), the purified enzyme solution (1.2 ml, 7.0 U/ml) and 6% inulin (1.5 ml) were mixed. The enzyme reaction was performed at 50 °C, and periodically (1, 4, 24 h) a part of reaction mixture was sampled. The obtained sample was heated at 100 °C for 7 min to stop the reaction. 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.

2.9. Preparation of reaction products from fructo-oligosaccharides

0.1 M citrate buffer, pH 5.5 (0.2 ml), fructo-oligosaccharide (GF_2 or GF_3 or GF_4, 10%, 0.3 ml) and the enzyme solution (0.5 ml, 7 U/ml) were mixed and reacted at 50 °C for 24 h. After the reaction the mixtures were heated at 100 °C for 7 min to stop the enzyme reactions. After cooling, the reaction mixtures were analyzed by a paper chromatography with the same method mentioned in Section 2.8.

3. Results and discussion

3.1. Identification of the microorganism

Table 1 summarized the results of taxonomic study. The microorganism D13-3 was a Gram-positive non-spore forming bacterium. It was catalase positive and oxidase negative. Therefore, the strain D13-3 was estimated to be a coryneform bacterium. The 16S rDNA sequence showed homology of 99.8% with that of *Arthrobacter ureafaciens* (type strain). And on the molecular genealogical analysis of the 16S rDNA sequence, the strain D13-3 formed a same cluster with that of *Arthrobacter ureafaciens* (data not shown). From these results, the strain D13-3 was identified as *Arthrobacter ureafaciens* D13-3.

Table 2
Purification of inulin fructotransferase (DFA III-producing) from *Arthrobacter ureafaciens* D13-3.

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)
Crude enzyme	1400	108	13.0	100
DEAE-Toyopearl	851	6.07	140	60.8
Super Q-Toyopearl (1st)	621	2.29	271	44.4
Super Q-Toyopearl (2nd)	331	1.14	290	23.6

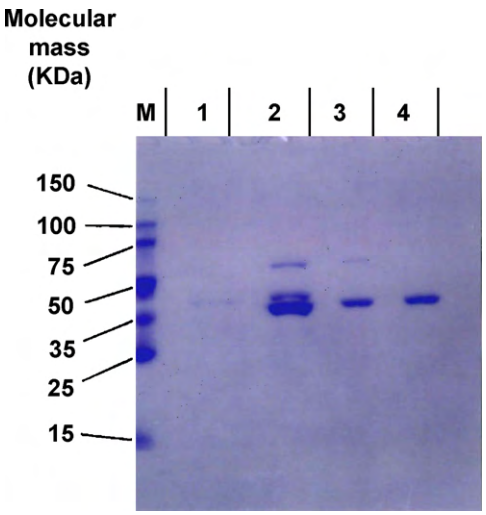

Fig. 1. The SDS-PAGE of the intermediate fractions and the purified enzyme. Lane M: molecular mass standard markers; lane 1: crude enzyme; lane 2: DEAE-Toyopearl fraction; lane 3: Super Q Toyopearl fraction (1st); lane 4: Super Q Toyopearl Fraction (2nd) (purified enzyme).

3.2. Purification of enzyme

Table 2 showed a summary of the enzyme purification. The enzyme was purified 22.3-fold with a yield of 23.6% by a DEAE-Toyopearl chromatography and two times of Super Q Toyopearl chromatography. The intermediate fractions of purification and the purified enzyme was analyzed by SDS-PAGE, the Super Q Toyopearl (2nd) fraction gave a single band (Fig. 1).

3.3. Effect of pH and temperature on enzyme activity

The effect of pH on the enzyme activity was investigated in the pH range 4.0–8.0 at 50 °C. As shown in Fig. 2(A), maximum activity was obtained at pH5.5. The enzyme reaction was performed in the range 30–75 °C at pH5.5. As shown in Fig. 2(B), maximum activity was obtained at 50 °C.

3.4. Thermal stability

The enzyme solution was heated at various temperatures for 30 min at pH 5.5, after that the residual activities were measured at pH 5.5 and 50 °C. As shown in Fig. 2(C), the enzyme was stable up to 70 °C, but it was inactivated at 75 °C.

3.5. 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 40 kDa. Fig. 3(B) shows the result of molecular mass estimation by gel filtration with TSK-gel G3000SWXL. The molecular mass was estimated to be 43 kDa. From these results, the enzyme was considered to be a monomer. Table 3 summarizes the comparison of some properties of inulin fructotransferases (DFAIII-producing) from different microorganisms.

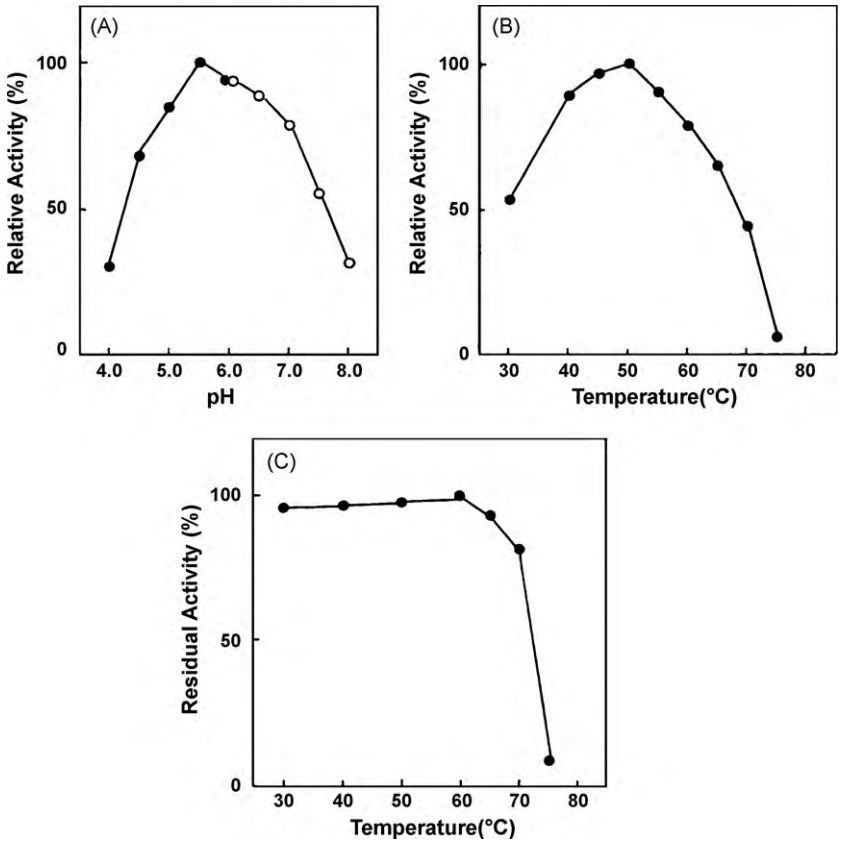

Fig. 2. (A) The effect of pH on the enzyme activity: (●) citrate buffer; (○) phosphate buffer. (B) The effect of temperature on the enzyme activity. (C) The thermal stability of the enzyme.

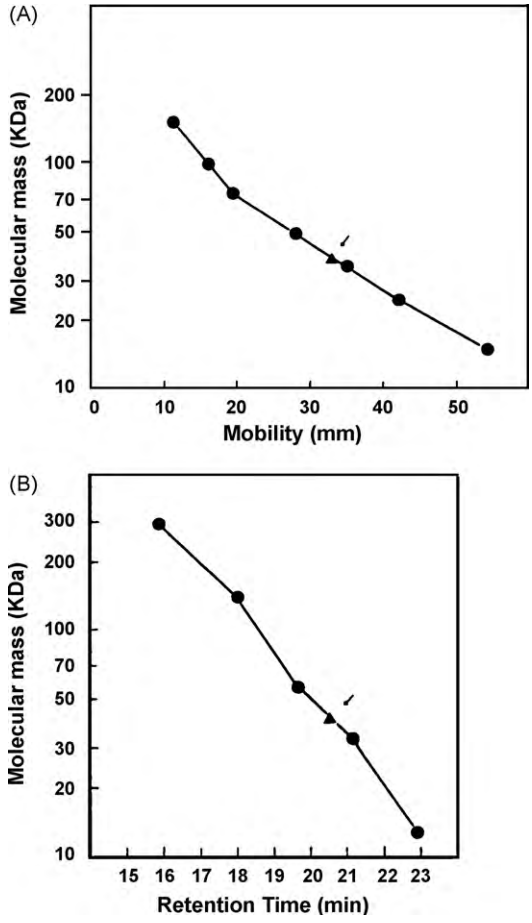

Fig. 3. (A) The estimation of the molecular mass by SDS-PAGE. Standard marker proteins, Takara perfect protein markers (150, 100, 75, 50, 35, 25, 15 kDa). (B) The estimation of the molecular mass by gel filtration. Standard marker proteins, glutamate dehydrogenase (290 kDa); lactate dehydrogenase (142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

3.6. N-terminal amino acid sequence

N-terminal amino acid sequence (12 amino acid residues) was determined as TTVYDVTTVDVP. Fig. 4 shows the comparison of N-terminal amino acid sequence of inulin fructotransferases (DFAIII-producing) from various microorganisms. At the N-terminal amino acid sequence of *A. ureafaciens* D13-3, the 11 amino residues were deleted compared with those of *A. globiformis* C11-1 (Haraguchi et al., 1988), *Bacillus* sp. Snu-7 (Kang et al., 1998), and *A. pascens* T13-2 (Haraguchi et al., 2002).

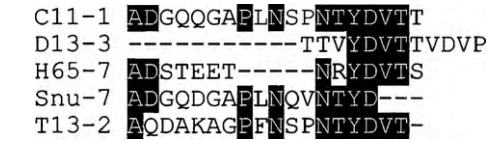

Fig. 4. The comparison of N-terminal amino acid sequence of inulin fructotransferase (DFAIII-producing) from different microorganism. C11-1: the sequence of *A. globiformis* C11-1; D13-3: the sequence of *A. ureafaciens* D13-3; H65-7: the sequence of *Arthrobacter* sp. H65-7; Snu-7: the sequence of *Bacillus* sp. Snu-7; T13-2: the sequence of *A. pascens* T13-2. The identical residues are presented by white letter in black boxes.

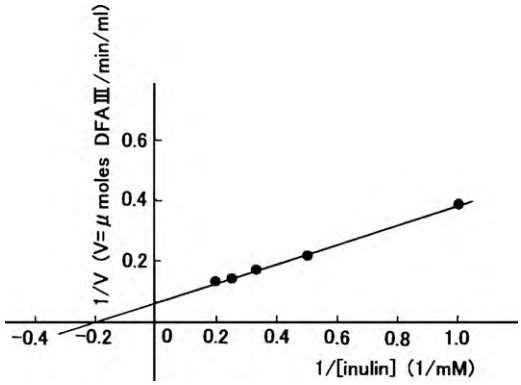

Fig. 5. The estimation of K_m value of the enzyme by double reciprocal plots.

3.7. Estimation of K_m value

The enzyme reaction was performed at pH 5.5 and 50 °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 $-1/K_m$ (-1/mM) value at the conditions was estimated to be -0.2. Therefore the K_m value at the condition was estimated to be 5 mM.

3.8. Reaction products from inulin

The reaction mixtures of the enzyme from *A. ureafaciens* D13-3, using an inulin as a substrate, were analyzed by paper chromatography. As shown in Fig. 6, the minor products after the 24 h enzyme reaction were GF, GF_2 and GF_3. Mostly, the minor products of the inulin fructotransferase (DFA III-producing) were GF_3 and GF_4 (Haraguchi et al., 1988, 2002, 2005, 2006; Kawamura et al., 1988; Yokota et al., 1991). Kang et al. (1998) reported that the minor prod-

Table 3
The comparison of properties of inulin fructotransferases (DFA III-producing) from different microorganisms.

Microorganism	Optimum		Heat stability (°C)	Molecular mass (kDa)		K_m (mM)	References
	pH	°C		SDS-PAGE	Gel filtration		
A. ureafaciens D13-3	5.5	50	70	40	43	5	This work
Leifsonia sp. T88-4	5.0	65	60	44	74	1	Haraguchi et al. (2006)
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	0.8	Yokota et al. (1991)
A. pascens T13-2	5.5–6.0	50	75	44	79		Haraguchi et al. (2002)
Arthrobacter sp.L68-1	5.5–6.0	55	80	43	73	10	Haraguchi et al. (2005)
Bacillus sp. snu-7	6.0	40	60	62		5.4	Kang et al. (1998)

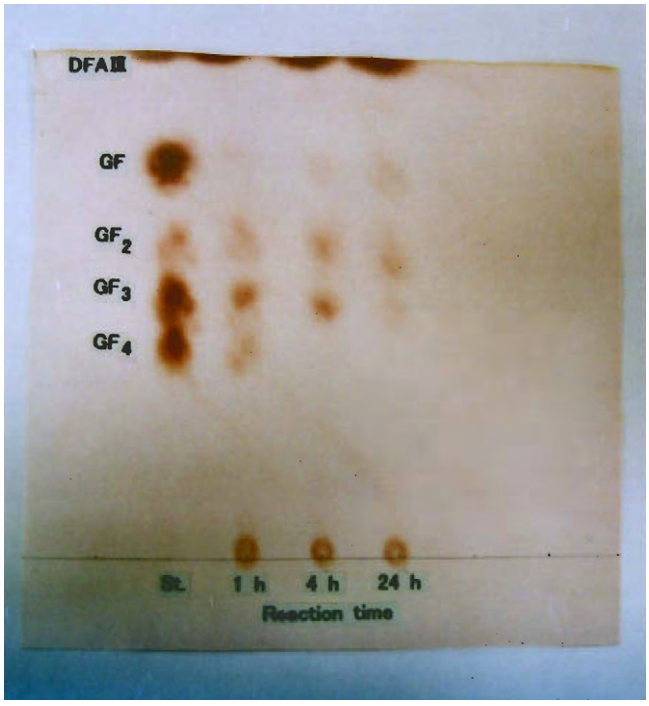

Fig. 6. The paper chromatogram of reaction products from inulin. GF: sucrose; GF_2: 1-kestose; GF_3: nystose; GF_4: fructosyl nystose; St.: Standards.

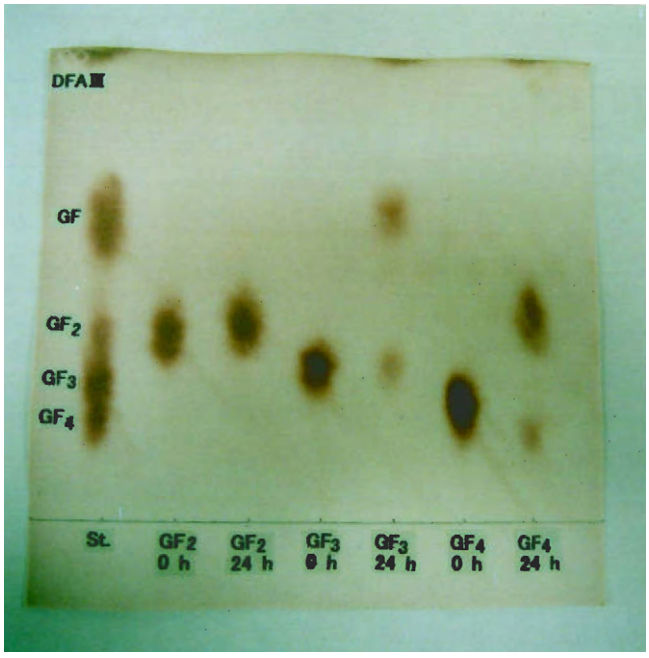

Fig. 7. The paper chromatogram of reaction products from fructo-oligosaccharide. GF: sucrose; GF_2: 1-kestose; GF_3: nystose; GF_4: fructosyl nystose; St.: Standards.

ucts of the enzyme from *Bacillus* sp. were the GF_2, GF_3 and GF_4. Therefore, the minor products of the enzyme reaction (GF, GF_2 and GF_3) are the one of the unique characteristics of the enzyme from *A. ureafaciens* D13-3.

3.9. Reaction products from fructo-oligosaccharide

As shown in Fig. 7, the enzyme from *A. ureafaciens* D13-3 could not react on a GF_2. Though, a DFAIII and a residual GF (sucrose) was produced from a GF_3. And a DFAIII and a residual GF_2 (1-kestose) was produced from a GF_4. From these results, it was estimated that the smallest fructo-oligosaccharide as the substrate of the enzyme of *A. ureafaciens* D13-3 was the GF_3 (nystose).

4. Conclusion

The inulin fructotransferase (DFAIII-producing) of *Arthrobacter ureafaciens* D13-3 was purified and characterized. The enzyme showed maximum activity at pH 5.5 and 50 °C. The enzyme was stable up to 70 °C for 30 min heat treatment. The molecular mass of the enzyme was estimated to be 43 kDa by SDS-PAGE and 40 kDa by gel filtration. Therefore, the enzyme was considered to be a monomer. The smallest fructo-oligosaccharide as the substrate was estimated to be the GF_3 (nystose).

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