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Short communication

Cloning of inulin fructotransferase (DFA III-producing) gene from *Arthrobacter* sp. L68-1☆

Kazutomo Haraguchi*

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

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ABSTRACT

A gene of inulin fructotransferase (DFA III-producing) [EC 2.4.1.93] from *Arthrobacter* sp. L68-1 was cloned and the nucleotide was sequenced. The gene encoded a signal peptide (32 amino acid residues) for a secretion, and the mature enzyme protein was estimated to be consisted with 410 amino acid residues. The molecular mass of the native enzyme was calculated as 43.7 kDa by the sequence data. The deduced amino acid sequence of the enzyme had 79.0% homology with that of the *Arthrobacter globiformis* C11-1, and had 77.4% homology with that of the *Arthrobacter* sp. H65-7. It also had 43.7% homology with that of inulin fructotransferase (DFA I-producing) [EC 2.4.1.200] from *A. globiformis* S14-3. The cloned enzyme was immobilized using Chitopearl BCW 3510 as a carrier. The immobilized enzyme was able to use 10 times without a significant loss of the enzyme activity.

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, 2010; 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).

On the cloning of inulin fructotransferase (DFA III-producing) gene, that of *Arthrobacter* sp. H65-7 was reported (Sakurai, Yokota, & Tomita, 1997). We reported on the gene from *Arthrobacter globiformis* C11-1 (Haraguchi, Mori, & Hayashi, 2000).

The DFA III 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 DFA III has a potential for the improvement of osteoporosis and iron deficiency anemia. The sales of DFA III started in 2004 in Japan.

In our previous reports (Haraguchi et al., 2005), we reported on the heat stable inulin fructotransferase (DFA III-producing) from *Arthrobacter* sp. L68-1. The heat-stability of this enzyme (80 °C, 60 min) was highest reported to date. In this paper, we describe the cloning, the nucleotide sequencing, and the immobilization of the cloned enzyme from *Arthrobacter* sp. L68-1.

2. Materials and methods

2.1. Purification of native enzyme

Inulin fructotransfersse (DFAIII-producing) was purified from the culture supernatant of *Arthrobacter* sp. L68-1 using the method described in our previous report (Haraguchi et al., 2005).

2.2. Partial digestion of enzyme protein by lysyl endopeptidase

The purified enzyme protein (0.3 mg) was dissolved in 0.2 ml of 0.1 M Tris–HCl buffer, pH 9.0, containing 0.2% SDS. The mixture was heated at 100 °C for 5 min to denature the protein. After cooling, lysyl endo-peptidase from *Achromobacter lyticus* M497-1 (20 μg,

☆ The DNA sequence data presented in this paper will appear in the DDBJ/ EMBL/ Genbank DNA databases with the accession number AB705454.

* Corresponding author. Tel.: +81 029 838 8073; fax: +81 029 838 7996.
E-mail address: haraguti@affrc.go.jp

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Wako Pure Chemical Co. Ltd., Osaka) was added, and reacted at 30 °C for 19 h. The reaction was stopped by heating at 100 °C for 5 min. The reaction mixture was separated by SDS-PAGE using a ready made gel (SPU-15S, Atto Co. Ltd., Japan) and blotted on a PVDF membrane (Sequi-Blot, Bio-Rad Co. Ltd., USA). The bands of the lysyl endo-peptidase fragment were subjected to amino acid sequencing.

2.3. Amino acid sequencing

Amino acid sequence of N-terminal region of the enzyme and lysyl endo-peptidase fragments were analyzed by automated Edman degradation with a G1005 peptide sequencer (Hewlett Packard Co. Ltd., USA).

2.4. Preparation of chromosomal DNA

Arthrobacter sp. L68-1 was cultivated in a 500 ml shaking flask containing 100 ml of a medium at 30 °C for 24 h. The composition of the medium was described in our previous report (Haraguchi et al., 2005). After cultivation the cells were harvested by a centrifugation (6 000 × *g*, 20 min). The chromosomal DNA of the microorganism was extracted using the DNA extraction kit ISOPLANT II (Nippon Gene Co. Ltd., Japan).

2.5. PCR reaction 1 (amplification of a part of the gene)

The two mixed primers were chemically synthesized. The polymerase chain reaction (PCR) product was obtained with a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems Co. Ltd., USA). The reaction mixture (50 μl) consisted of 10× PCR buffer (5 μl), the chromosomal DNA of *Arthrobacter* sp. L68-1 (1 μg), primers (1 μM), dNTPs (200 μM), and Takara *Taq* DNA polymerase (2U, Takara-Bio Co. Ltd., Japan). The PCR reaction was performed for 40 cycles. One cycle consisted of treatment at 94 °C for 0.5 min, at 40 °C for 1 min, and at 72 °C for 1 min.

2.6. Hybridization

For Southern hybridization and colony hybridization, hybond N^+ membranes (GE Healthcare Co. Ltd., USA) were used. The hybridization probes were labeled using a non-RI labeling kit (GeneAmp Alkphos direct labeling and detection systems, GE Healthcare Co. Ltd., USA). The hybridization was performed in a buffer of the labeling kit at 55 °C.

2.7. DNA sequencing

DNA fragments were digested by various restriction enzymes and sub-cloned into plasmid pUC118 or pUC119. The sequencing reaction was performed with Big-dye terminator cycle sequencing kit v1.1 (Applied Biosystems Co. Ltd., USA) using a thermal cycler (GeneAmp PCR system 9700, Applied Biosystems Co. Ltd., USA). The nucleotide sequence was determined with 310 Genetic Analyzer (Applied Biosystems Co. Ltd., USA).

2.8. PCR reaction 2 (amplification of the whole gene)

The two primers were chemically synthesized (Forward: 5′ GGTTCATTTGGGTGACAA 3′, Reverse: 5′ TTGAGGCGTCAGGGTGTA 3′). The PCR product was obtained with the thermal cycler (GeneAmp PCR System 9700, Applied Biosystems Co. Ltd., USA). The reaction mixture (50 μl) consisted of 10× PCR buffer (5 μl), the chromosomal DNA of *Arthrobacter* sp. L68-1 (0.2 μg), primers (0.8 μM), dNTPs (200 μM), and Takara *Taq* polymerase (5U, Takara-Bio Co. Ltd., Japan). The PCR reaction was performed for 30 cycles. One cycle consisted of treatment at 94 °C for 0.5 min, at 45 °C for 0.5 min, and 72 °C for 1 min.

2.9. Standard enzyme assay

0.1 M phosphate buffer, pH 6.0 (0.5 ml), the enzyme solution (0.1 ml), and water (0.4 ml) were mixed. The reaction was started with the addition of 2% inulin (1.0 ml) and reacted for 30 min at 55 °C. The reaction was stopped by heating at 100 °C for 7 min. After cooling, the amount of DFA III produced was determined with HPLC (column; Shimpack CLC ODS, Shimadzu Co. Ltd., Japan, mobile phase; water, detector; refractive index detector). One unit of the enzyme was defined as which can produce 1 μmol DFA III per min at pH 6.0 and 55 °C.

2.10. Preparation of cloned enzyme solution

One loop of the *Escherichia coli* JM109 carrying a plasmid (pL68-1, containing the enzyme gene) was inoculated in a test tube containing 3 ml of a medium (L-broth; 0.1% Tryptone, 0.05% yeast extract, 0.1% NaCl, pH 7.0, containing 0.1 mg/ml ampicillin) and cultured for 4 h at 37 °C. This pre-culture (0.3 ml) was inoculated in a test tube containing 3 ml of the same medium and cultured for 2 h at 37 °C. The 10 mM IPTG (Isopropyl-beta-D (−) thiogalactopyranoside, 0.15 ml) was added to the culture, and cultured for 18 h at 37 °C. The five tubes (cultures) were prepared. The cells (totally 14 ml culture) were harvested with a centrifugation (5000 × *g*, 15 min) and washed once with 0.1 M phosphate buffer, pH 6.0. The cells were suspended in 4 ml of 0.1 M phosphate buffer, pH 6.0, and the cells were disrupted by a sonication (5 min, Sonifier 450, Branson Co. Ltd., USA). The solution was centrifuged (8000 × *g*, 20 min) and the supernatant was used as a cell extract (cloned enzyme solution).

2.11. Preparation of immobilized enzyme

The cloned enzyme solution (3 ml, 1.04 units/ml), 0.1 M phosphate buffer, pH 6.0 (6.0 ml), and Chitopearl BCW3510 (5.0 g; Fuji Boseki Co. Ltd., Japan) were mixed with shaking at room temperature for 1 h. The prepared immobilized enzyme was washed thoroughly with water. To stabilize the immobilized enzyme, 10 ml of 2.5% glutaraldehyde was added, and mixed with shaking at room temperature for 1 h. The sample was washed thoroughly with water and used as an immobilized cloned enzyme.

2.12. Reaction using immobilized enzyme

The immobilized cloned enzyme (2.0 g) was put in an Erlenmeyer flask (50 ml). The reaction was started with the addition of 0.1 M phosphate buffer, pH 6.0, containing 1% inulin (5.0 ml). The reaction was performed at 55 °C with shaking for 30 min. After reaction, the reacted mixture was moved to a test tube and heated at 100 °C for 7 min to stop the reaction. After cooling, the DFA III produced was determined by HPLC (as mentioned in Section 2.9).

3. Results and discussion

3.1. PCR reaction and preparation of hybridization probe

The N-terminal amino acid sequence of the native enzyme was AEETKGGPFNSPNAYDVT- and that of a lysyl endopeptidase fragment was (K)TGIDVAS-. The two mixed primers (primers

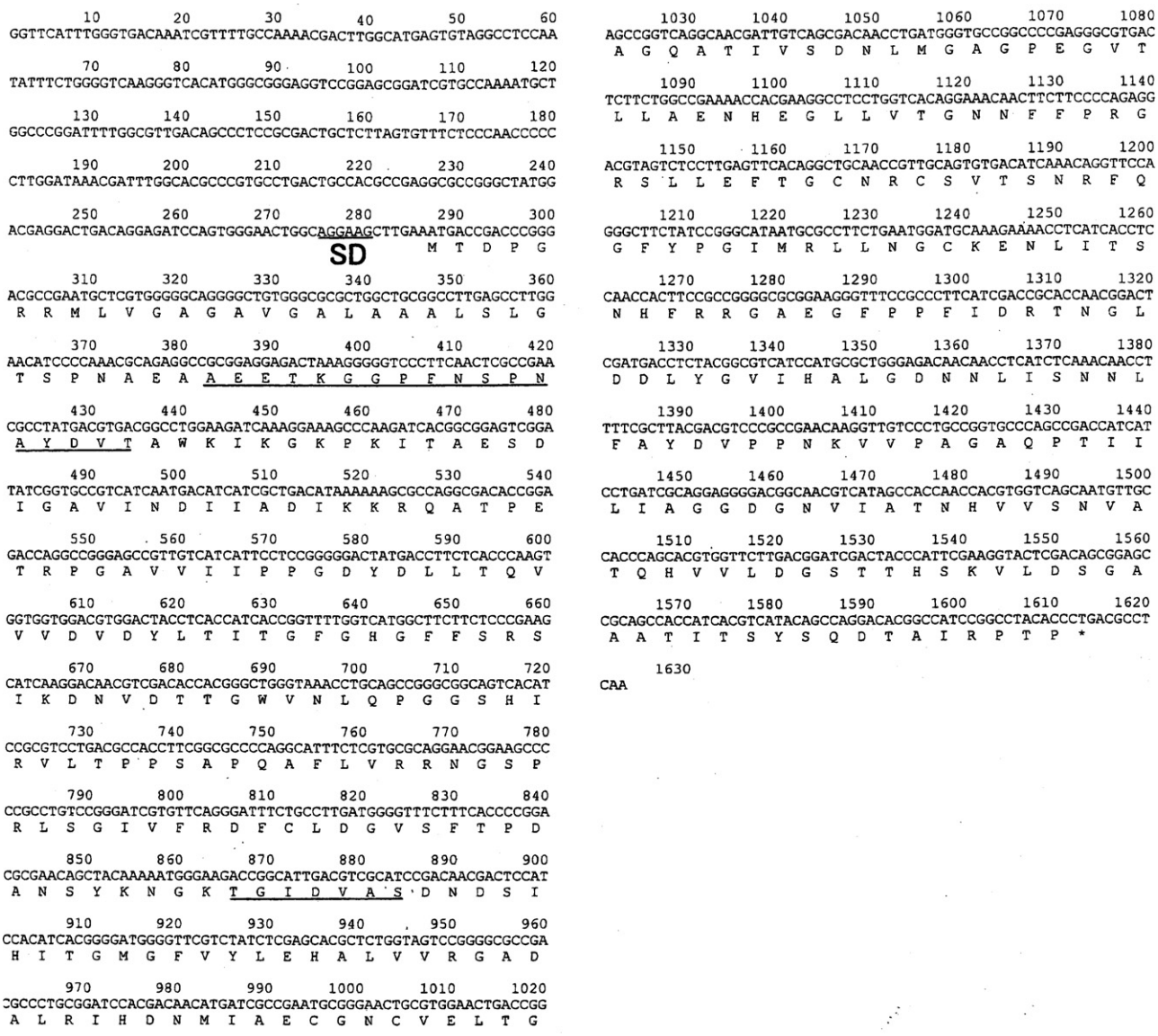

Fig. 1. Structure of chromosomal fragment containing inulin fructotransferase (DFA III-producing) gene from *Arthrobacter* sp. L68-1. The putative ribosomal biding site (SD) is underlined. The amino acid sequence of the N-terminal region of the native enzyme and the lysyl endo-peptidase fragment are underlined.

F and RV) were chemically systhesized based on the sequence of the native enzyme and the lysyl endopeptidase fragment (Primer F: 5′ AA(CT)GC(ACGT)TA(CT)GA(CT)GT(ACGT)AC 3′, Primer RV: 5′ AC(AG)TC(AGT)AT(AGTC)CC(ATGC)GT(CT)TT 3′). Using these primers, a PCR reaction was performed, and a 0.5-kb fragment was amplified. This fragment was purified by agarose electrophoresis and recovered using DNA Cell (Daiichi Kagaku Yakuhin Co. Ltd., Japan). The recovered PCR product was labeled by the non-RI kit (Section 2.6), and used as a probe for the hybridizations.

3.2. Cloning of enzyme gene

The chromosomal DNA of *Arthrobacter* sp. L68-1was digested with *Kpn* I and *Sca* I. The Southern Hybridization was performed, and the probe was hybridized with a 2.8-kb fragment. The *Kpn* I and *Sca* I digests of the chromosomal DNA of *Arthrobacter* sp. L68-1 were electrophoresed on an agarose gel and a 2.8-kb fraction was recovered. The fraction was ligated into the *Kpn* I, *Hinc* II site of pUC 118. Using this ligation mixture, *E. coli* HST08 was transformed and a gene library was constructed. The gene library was screened by colony hybridization and a positive clone was obtained, and the nucleotide sequence was determined.

For the expression of the gene in *E. coli*, a short fragment containing the whole gene is desirable. As shown in Section 2.8, the 1.6-kb fragment containing the inulin fructotransferase (DFA III-producing) gene was amplified by a PCR reaction, and ligated with pGEM-T vector (Promega Co. Ltd., USA). Using this plasmid, *E. coli* JM109 was transformed. The plasmid obtained from this clone was digested with *Sac I* and *Sph* I. The obtained 1.6-kb *Sac* I, *Sph* I fragment was purified by agarose electrophoresis, and introduced at a *Sac* I/*Sph* I site of pUC 119. This plasmid was designated as pL68-1. In this plasmid, the enzyme gene is downstream of *lac* promoter of pUC119.

3.3. Nucleotide sequence of enzyme gene

The nucleotide sequence of the cloned 1.6-kb fragment is shown in Fig. 1. The fragment contains an open reading frame consisted by 1329 bp. As mentioned previously, the N-terminal amino acid sequence of the native enzyme was

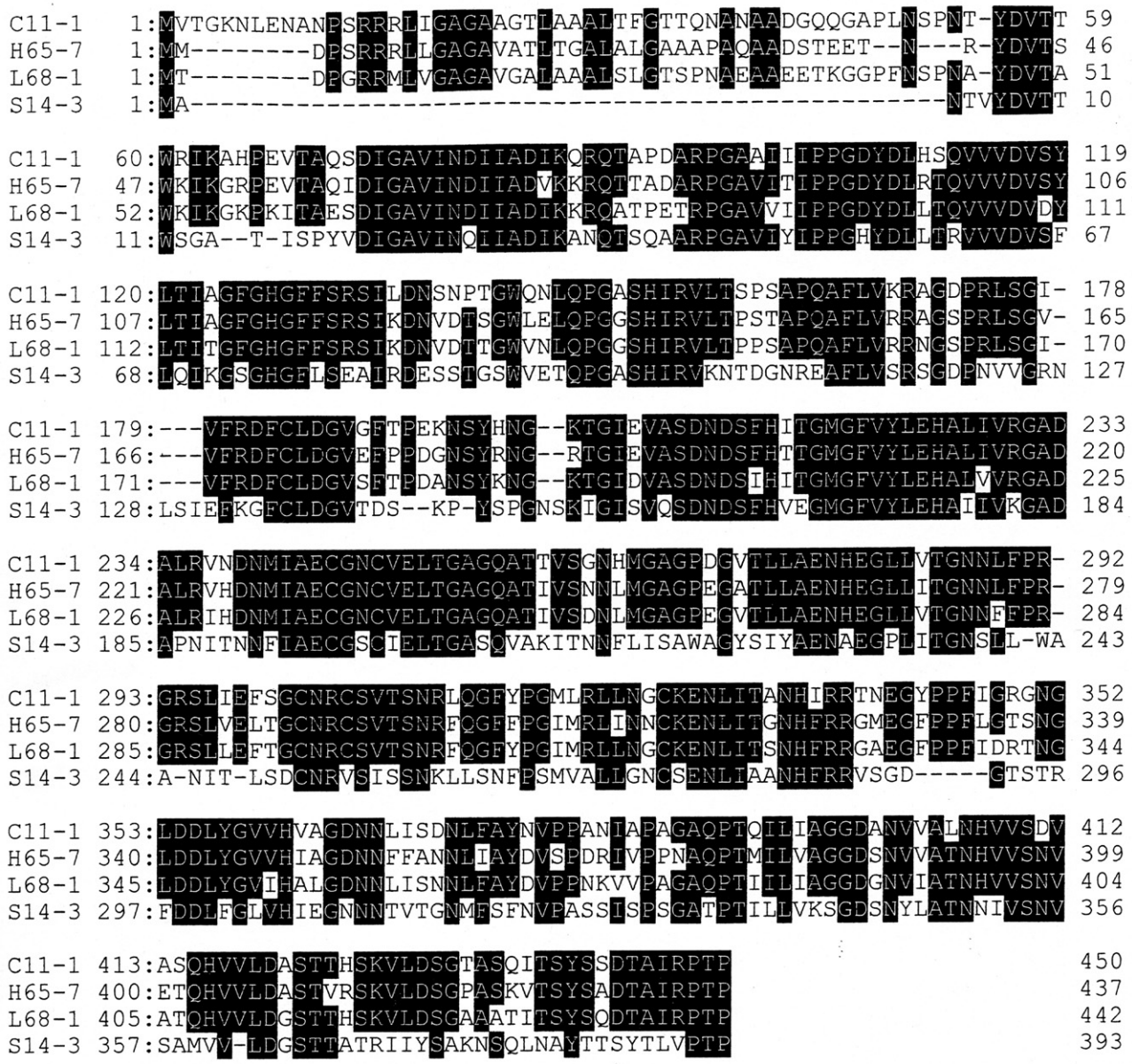

Fig. 2. Alignment of the deduced amino acid sequence of inulin fructotransferases. C11-1, deduced sequence of inulin fructotransferase (DFA III-producing) from *Arthrobacter globiformis* H65-7; deduced sequence of the enzyme from *Arthrobacter* sp. H65-7; L68-1, deduced sequence of the enzyme from *Arthrobacter* sp. L68-1; S14-3, deduced sequence of inulin fructotransferase (DFA I-producing) from *A. globiformis* S14-3. The identical residues are presented by white letter in black boxes.

AEETKGG-. Therefore the gene encoded a signal peptide (32 amino acid residue), and the mature enzyme protein consisted by 410 amino acid residues. The molecular mass of the native enzyme (mature enzyme) was calculated as 43.7 kDa from the DNA sequence data. In our previous report (Haraguchi et al., 2005), we reported that the molecular mass was estimated to be 43 kDa by SDS-PAGE. The molecular mass obtained by DNA sequencing does not contradict with the former datum.

Fig. 2 shows the homology of deduced amino acid sequence of various inulin fructotransferases. The deduced amino acid sequence of inulin fructotransferase (DFA III-producing) of *Arthrobacter* sp. L68-1 had 79.0% homology with that of *A. globiformis* C11-1 (Haraguchi et al., 2000), and had 77.4% homology with that of *Arthrobacter* sp. H65-7 (Sakurai et al., 1997). It also had 43.7% homology with that of inulin fructotransferase (DFA I-producing) from *A. globiformis* S14-3 (Haraguchi et al., 1995).

3.4. Reaction using immobilized enzyme

The cell extract and the immobilized enzyme were prepared as in Sections 2.11 and 2.12. The prepared cell extract (4 ml) showed an activity of 1.04 units/ml (The activity of culture supernatant of *Arthrobacter* sp L68-1 was 8 units/ml). The immobilized enzyme was prepared using 3 ml of the cell extract. The prepared immobilized enzyme (5 g) showed an activity of 0.32 units/g. Therefore, the recovery of enzyme activity at the immobilization was calculated as 100•(0.32•5)/(1.04•3) = 51.3%. Fig. 3 shows the repeated use of immobilized enzyme. As shown in Fig. 3, the immobilized enzyme was able to use 10 times without a significant loss of the activity.

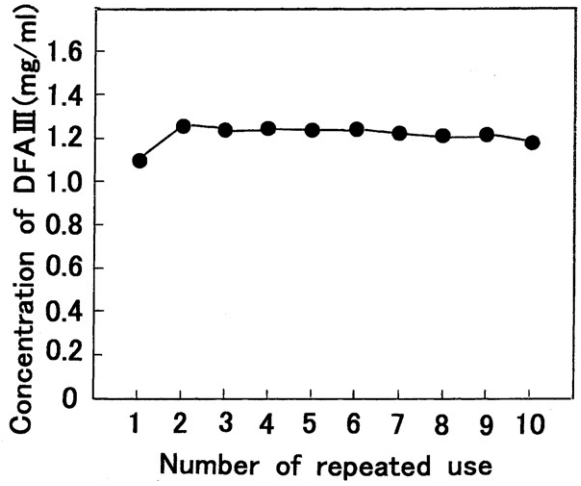

Fig. 3. Repeated use of immobilized cloned enzyme.

4. Conclusions

The gene of inulin fructotransferase (DFA III-producing) from *Arthrobacter* sp. L68-1 was cloned and the nucleotide sequence was determined. The enzyme gene coded a signal peptide (32 amino acid residues) for secretion. The native enzyme was estimated to be consisted with 410 amino acid residues. The molecular mass of the native enzyme was calculated as 43.7 kDa from the sequence data. The deduced amino acid sequence of the enzyme had 79.0% homology with that of *A. globiformis* C11-1 and it had 77.4% homology with that of *Arthrobacter* sp. H65-7. It also had 43.7% homology with that of inulin fructotransferase (DFA I-producing) gene from *A. globiformis* S14-3. The immobilized cloned enzyme was prepared using Chitopearl BCW3510 as a carrier. The immobilized enzyme was able to use 10 times without a significant loss of the activity.

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