JARQ 32, 1-5 (1998)

Cloning of the Gene of DFA I Oligosaccharide-Producing Enzyme

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Abstract

DFA I (difructose dianhydride I) is a disaccharide composed of 2 molecules of fructose. DFA I is generated from inulin by the action of an enzyme produced by a bacterium *Arthrobacter globiformis* S14-3. The gene encoding the enzyme was cloned and the nucleotides sequenced, for the first time. The sequence indicated that the native enzyme is composed of 392 amino acid residues. The 1.5 kb DNA fragment encoding the gene was found to produce the active enzyme, under the control of the lac promoter of pUC 119.

Discipline: Biotechnology
Additional key words: gene engineering, inulin

Introduction

Sugar beet is an important crop for sugar production. In recent years, the consumption of sugar in Japan and European countries has reached a plateau, because of the spread of high fructose corn syrup and the popularity of low calorie sweeteners among consumers. In the European countries, chicory is being introduced as an alternative crop for sugar beet, because chicory can be cultivated using the same agricultural machines as those for the cultivation of sugar beet. Presently, chicory is mainly utilized as a vegetable. On the other hand the root of chicory contains a large amount of inulin.

Inulin is a storage polysaccharide contained in chicory, Jerusalem artichoke, etc. with a chemical structure consisting of beta-2,1 linked fructose polymer with a terminal sucrose residue. In studies on inulin-decomposing enzymes, hydrolases (inulinase) from yeast[1,3] and mold[2] were mainly reported. Uchiyama et al.[5] identified a new type of inulin-decomposing enzyme. The enzyme converted inulin into DFA III (di-D-fructofuranose 1, 2': 2, 3' dianhydride) and a small amount of oligosaccharides. The enzyme was designated as inulin fructotransferase (DFA III-producing) [EC 2. 4. 1. 93].

We isolated a bacterium which produced a novel type of inulin-decomposing enzyme[4]. The enzyme converted inulin into DFA I (di-D-fructofuranose 1, 2' : 2, 1' dianhydride) and a small amount of nystose (GF_3) and fructofuranosyl nystose (GF_4). The enzyme was designated as inulin fructotransferase (DFA I-producing), which was officially recognized as [EC 2. 4. 1. 200]. The bacterium which produces this enzyme was identified as *Arthrobacter globiformis* S14-3. Since DFA I is half as sweet as sucrose, it can be used as a low calorie sweetener. In this paper, we describe the cloning of the gene of the enzyme and the nucleotide the sequence of the gene.

Materials and methods

1) *Purification of enzyme*

Inulin fructotransferase (DFA I-producing) was purified from a culture supernatant of *Arthrobacter globiformis* S14-3 by the method described in the previous report[4].

2) *Partial digestion of enzyme protein by lysyl endopeptidase*

The purified enzyme protein was digested with lysyl endopeptidase for 20 h at pH 9.5 and 30°C. The peptidase reaction was stopped by heating at 100°C for 5 min. The reaction products were subjected to high pressure liquid chromatography on a Shimpack CLC ODS column, and eluted with a linear gradient of acetonitrile (0 to 80%) containing 0.1% trifluoroacetic acid.

3) *Amino acid sequencing*

The amino acid sequences of the N-terminal region of the enzyme and the lysyl endopeptidase fragment were analyzed with a Perkin Elmer 477A/120A protein sequencer.

4) *PCR reaction*

Mixed oligonucleotide primers were chemically synthesized. The PCR product was obtained with a DNA thermal cycler (Zymoreactor II, Atto Co., Ltd.) using Ampli Taq DNA polymerase. The PCR reaction was conducted for 40 cycles, with 1 cycle at 94°C for 0.5 min, 40°C for 2 min, 72°C for 3 min.

5) *DNA sequencing*

The DNA fragments were digested with various restriction enzymes and subcloned into plasmids pUC 118 or pUC 119 and, using the helper phage M13K07, single strand DNA for sequencing was prepared. The dideoxy sequencing reaction was performed with Ampli Taq DNA polymerase using a Prism Dye Primer Unicycle Sequencing Kit (Perkin Elmer Co., Ltd.). The nucleotides were sequenced with a 373A DNA sequencer (Perkin Elmer Co., Ltd.).

6) *Expression of inulin fructotransferase (DFA I-producing) gene in* E. coli

For the preculture, *E. coli* clones were cultured overnight in a test tube containing Luria-Bertani broth with ampicillin (100 μg/ml) at 37°C. The cultures (0.1 ml) were inoculated into the same medium, containing 1 mM IPTG or without IPTG, and cultured at 37°C for 24 h. The cells were removed by centrifugation (10,000 × g, 15 min). The inulin fructotransferase activity in the culture supernatants was measured by the method described in our previous report[4]. For the preparation of the cell-free extract, the cells of the *E. coli* clone were washed with 10 mM phosphate buffer, pH 6.0, and resuspended in the same buffer. The cells were disrupted with a Sonifier 200 (Branson Co., Ltd.) and centrifuged (15,000 × g, 30 min). The supernatant was used as the cell-free extract and the enzyme activity was measured.

Probe1 5'GTGACCACTTGGTCTGGG3'

Probe2 5'TACGTCGACATCGGTGCT3'

Fig. 1. Synthesized hybridization probes

Results and discussion

1) *PCR reaction and preparation of probes*

The 2 mixed oligonucleotides were chemically synthesized based on the amino acid sequences of the N-terminal region of the enzyme and a lysyl endopeptidase fragment. Using these PCR primers, a 1-kb DNA fragment was amplified. The amplified DNA was purified by agarose electrophoresis and treated with a DNA Blunting Kit (Takara Shuzo Co., Ltd.). The blunted DNA was ligated at the Sma I site of pUC 118. *E. coli* MV1184 was transformed with the plasmid. The partial nucleotide sequence of the PCR product was analyzed and 2 probes were chemically synthesized (Fig. 1).

2) *Cloning of the enzyme gene*

The chromosomal DNA of *A. globiformis* S14-3 was digested with Sph I. The digests were analyzed by Southern hybridization. The hybridization was carried out at 46°C for probe 1, and 57°C for probe 2. In both cases, the probes hybridized with a 1.5 kb Sph I fragment. The Sph I digests of the chromosomal DNA were electrophoresed on an agarose gel and a 1.5 kb fraction was recovered with a DNA Cell (Daiichi Kagaku Yakuhin Co., Ltd.). The 1.5 kb fraction was ligated into the Sph I site of pUC 119. Using this ligation mixture, *E. coli* MV1184 was transformed and a gene library was constructed. The gene library was screened by colony hybridization and a positive clone was obtained. A plasmid obtained from this clone was designated as pIF1-1. Fig. 2 shows the restriction map of the cloned 1.5 kb fragment.

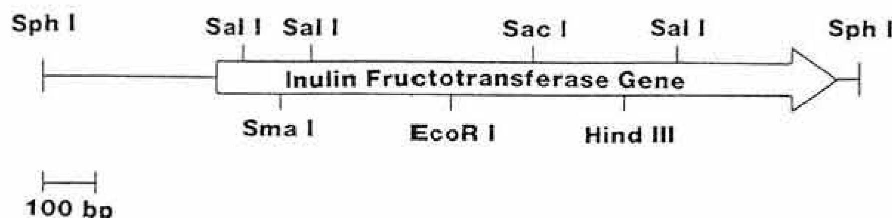

Fig. 2. Restriction map of cloned fragment

3) Nucleotide sequence of the gene

The nucleotide sequence of the cloned 1.5 kb fragment is shown in Fig. 3. The fragment contained a 1,182 bp open reading frame. The open reading frame started with ATG and ended with TAA. The N-terminal amino acid sequence of the native enzyme was described in our previous report[4] (NH_2-Ala-Asn-Thr-Val-Tyr...). Based on these results, it was estimated that the native enzyme was composed of 392 amino acid residues. The molecular weight of the enzyme was calculated to be 41,600. In our previous report[4], the molecular weight of the enzyme was estimated at 39,000 by SDS-PAGE and 46,000 by gel filtration. The molecular weight obtained from sequencing data was in agreement with the values described in our previous report. The native enzyme is an extracellar enzyme produced in a culture supernatant. Usually, an extracellular enzyme has a signal peptide for secretion, but in the nucleotide sequence of the enzyme gene, no sequence for signal peptide was found. The codon usage of the enzyme gene is shown in Table 1. The nucleotide sequence of the gene had no distinct homology with that of inulinase gene of *Kluyveromyces marxianus*[1].

4) Expression of the gene

In the plasmid pIF1-1, the gene of the enzyme was located downstream of the *lac* promoter of pUC 119. The cloned 1.5 kb fragment was introduced into pUC 119 in the opposite direction to that of pIF1-1. The constructed plasmid was designated as pIF1-2. The culture supernatant of the *E. coli* clone containing pIF1-1 or pIF1-2 was prepared, and the enzyme activity was measured. In the culture supernatant of the *E. coli* clone carrying pIF1-1 the enzyme activity was 0.34 U/ml when the culture medium contained 1 mM IPTG, while without IPTG the activity occurred as trace. However, the culture

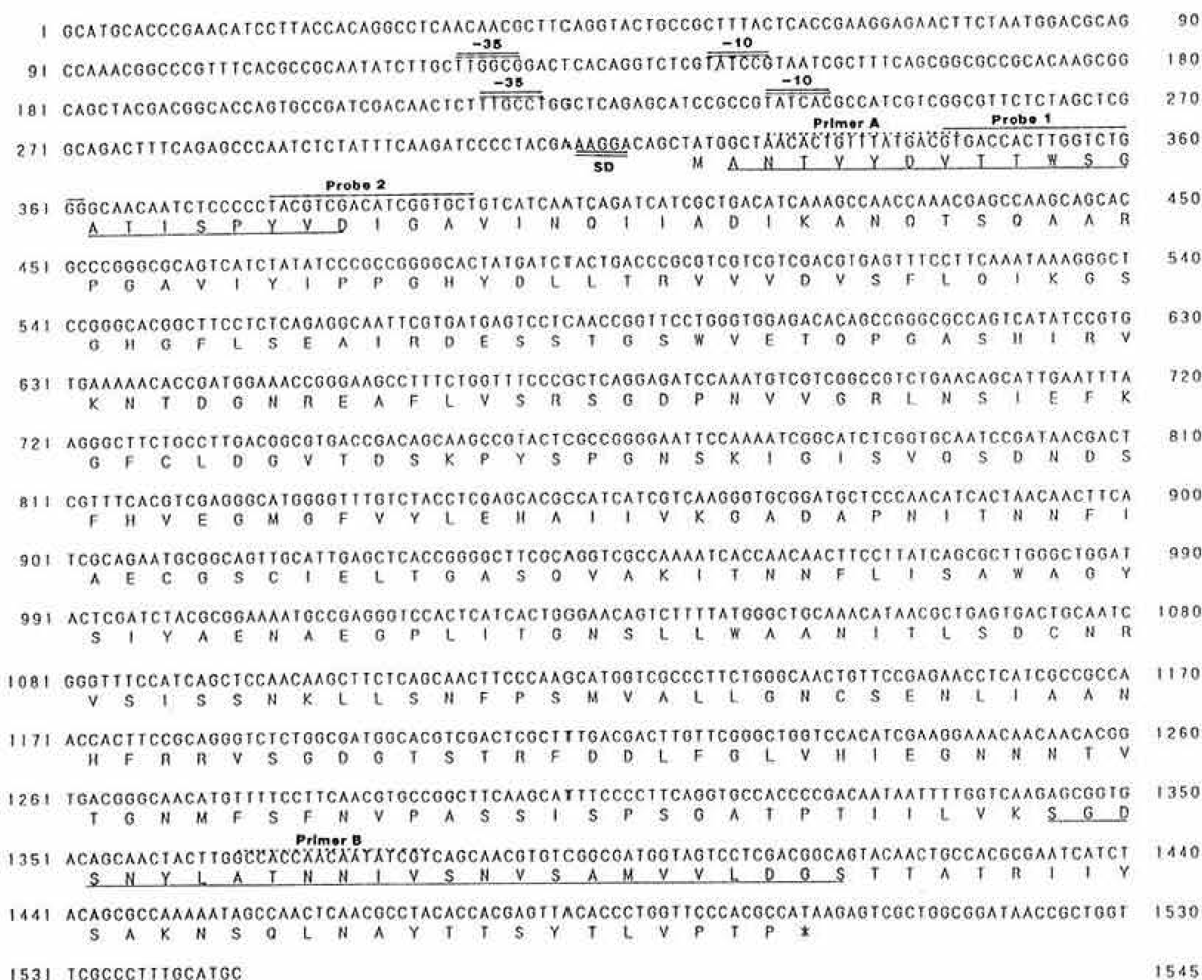

Fig. 3. Nucleotide sequence of chromosomal fragment containing the enzyme gene

Table 1. Codon usage of inulin fructotransferase (DFA I-producing)

Amino acid (Total)	Codon	Frequency of use	Amino acid (Total)	Codon	Frequency of use
Ala	GCT	9	Leu	CTG	7
(33)	GCC	14			
	GCA	7	Lys	AAA	5
	GCG	3	(11)	AAG	6
Arg	CGT	3	Met	ATG	5
(12)	CGC	5	(5)		
	CGA	1			
	CGG	2	Phe	TTT	6
	AGA	0	(15)	TTC	6
	AGG	1			
			Pro	CCT	1
Asn	AAT	7	(16)	CCC	3
(34)	AAC	27		CCA	4
				CCG	8
Asp	GAT	7			
(19)	GAC	12	Ser	TCT	2
			(46)	TCC	12
Cys	TGT	1		TCA	5
(5)	TGC	4		TCG	7
				AGT	7
Gln	CAA	5		AGC	13
(8)	CAG	3			
			Thr	ACT	6
Glu	GAA	5	(29)	ACC	11
(13)	GAG	8		ACA	4
				ACG	8
Gly	GGT	6			
(33)	GGC	15	Trp	TGG	4
	GGA	4	(4)		
	GGG	8			
			Tyr	TAT	3
His	CAT	1	(12)	TAC	9
(7)	CAC	6			
			Val	GTT	4
Ile	ATT	5	(32)	GTC	18
(33)	ATC	25		GTA	1
	ATA	3		GTG	9
Leu	TTA	1	Stop	TAA	1
(26)	TTG	3	(1)	TAG	0
	CTT	6		TGA	0
	CTC	8			
	CTA	1			

supernatant of the *E. coli* carrying pIF1-2 had no activity. The results suggest that the expression of the enzyme gene is controlled by the *lac* promoter of pUC 119. The cell-free extract of the *E. coli* clone (pIF1-1, with IPTG) was prepared and the enzyme activity was compared with that of the culture supernatant. The ratio of the total enzyme activity expressed (cell-free extract: culture supernatant) ranged from 46 to 54.

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(Received for publication, April 10, 1997)