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Purification and Properties of Cycloinulooligosaccharide Fructanotransferase from *Paenibacillus polymyxa* MG-CF6

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Abstract: A bacterial strain which produced a cycloinulooligosaccharide fructanotransferase was isolated from soil and was identified as *Paenibacillus polymyxa* MG-CF6. The enzyme was then purified to apparent homogeneity from culture supernatant of the bacterium. The molecular mass of the enzyme was estimated to be 128 kDa by SDS-PAGE. Maximal activity was found to be at pH 7.0 and 45°C. The enzyme was stable from pH 6.0 to 9.0 and at temperature up to 40°C. Molar ratio of cycloinulohexaose: cycloinuloheptaose formed by this enzyme was found to be about 2.5:1. N-Terminal amino acid sequence of the enzyme was identical with a part of amino acid sequence of the same enzymes from *Bacillus macerans* CFC1 and *Bacillus polymyxa* MGL 21. The gene of the enzyme was cloned from *P. polymyxa* MG-CF6. It consisted of 3999 nucleotides, which encodes a protein of 1333 amino acids. The deduced amino acid sequence showed 83, 95 and 98% similarity to that of *Bacillus circulans* MCI-2554, *Bacillus macerans* CFC1 and *Bacillus polymyxa* MGL21, respectively.

Key words: cycloinulooligosaccharide fructanotransferase, cycloinulooligosaccharide, inulin, cyclofructan, *Paenibacillus*

Cycloinulooligosaccharide fructanotransferase (CFTase) was discovered in 1989 by Kawamura *et al.*¹⁾ and was purified by Kawamura and Uchiyama.²⁾ This enzyme produces cycloinulooligosaccharides (CFs) from inulin, a linear β -(2-1)-linked polyfructofuranose chain having a terminal glucose residue. It has been reported that the CFs have the characteristic crown ether-like skeleton,³⁾ complexation properties with some metal ions,⁴⁾ and cryoprotective effects on the freeze-drying of liposome.⁵⁾ Thus, it is expected that CFs have a variety of function similar to cyclodextrins.

So far, it has been reported that only four bacteria, *Bacillus circulans* OKUMZ 31B,^{1,2)} *Bacillus circulans* MCI-2554,^{6,7)} *Paenibacillus* (*Bacillus*)^{**} *macerans* CFC1^{8,9)} and *Paenibacillus* (*Bacillus*)^{**} *polymyxa* MGL21,^{10,11)} produce CFTase. These enzymes were purified and their enzymatic properties were characterized. We also found a bacterial strain MG-CF6 which produced CFTase during our studies on enzymatic conversion of inulin into oligosaccharides and monosaccharides.

In this study, we report on identification of the bacterial strain as *Paenibacillus polymyxa* MG-CF6 and on purification of a CFTase, and its enzymatic properties. In addition, we also describe determination of the CFTase gene.

MATERIALS AND METHODS

Materials. Inulins from chicory roots, Jerusalem artichokes, and dahlia tuber were purchased from Sigma Co. (St. Louis, USA). Raftiline HP, which is spray-dried inulin prepared from chicory roots, was obtained from Orafit (Tienen, Belgium). Inulin (Fuji FF) which is produced from sucrose by a novel inulin-producing enzyme¹²⁾ was kindly supplied by Mr. M. Ohguchi, Fuji Nihon Seito Corporation (Shizuoka, Japan). Fructozyme L was purchased from Novozymes Japan Ltd. (Tokyo).

Cultivation. Cultivation medium composed of inulin (40 g/L), yeast extract (2 g/L), NaNO₃ (5 g/L), KH₂PO₄ (0.5 g/L), MgSO₄·7H₂O (0.5 g/L), KCl (0.5 g/L), FeCl₃ (10 mg/L), and CaCO₃ (1 g/L), in tap water (pH 7.0). The microorganism was cultivated at 35°C for 72 h on a rotary shaker (200 rpm).

Identification of Microorganism. Taxonomic characteristics of the bacterial strain MG-CF6 capable of producing CFTase were examined on the basis of Bergey's manual of systematic bacteriology.¹³⁾ The 16S rDNA sequence of the bacterium was also determined according to our method described previously.¹⁴⁾ Then, the 16S rDNA sequence determined was compared using the DNA data bank of Japan and the bacterium was identified.

Preparation of Cycloinulooligosaccharides. The microorganism was cultivated as described above and then culture broth (about 700 mL) was centrifuged at 18,500 × *g* for 10 min at 4°C. Solid ammonium sulfate was added to the supernatant to obtain 80% saturation. After standing overnight at 4°C, the precipitate was collected by

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Abbreviations: CFTase, cycloinulooligosaccharide fructanotransferase; CF, cycloinulooligosaccharide; CF6, cycloinulohexaose; CF7, cycloinuloheptaose.

** Genus of *Bacillus macerans* and *Bacillus polymyxa* has changed to genus *Paenibacillus* in taxonomy.

centrifugation and dissolved in about 10 mL of 10 mM sodium phosphate buffer (pH 7.0). The enzyme solution was then dialyzed with 1000 mL of the same phosphate buffer for 24 h at 4°C. The dialyzed solution was used as the crude enzyme preparation. This crude enzyme solution (3 mL) was added to 200 mL of 10% inulin (from chicory) solution and the mixture was incubated at 35°C for 60 h. To the reaction mixture was added Fructozyme L (800 U, Novozymes Japan Ltd.) containing both endo- and exo-inulinase, and was incubated for 1 h at 60°C. The solution was heated for 10 min in a boiling water bath. The reaction mixture was applied to a column (φ44×475 mm, 720 mL) of charcoal-Celite which had been equilibrated with distilled water. The column was washed with 3000 mL of distilled water and then eluted with 2100 mL of 30% ethanol solution. The effluent was evaporated and lyophilized to give 8.3 g of mixture containing two major compounds 1 and 2 by HPLC analysis as described below. The compounds 1 and 2 were isolated by QAE-Toyopearl 550C chromatography as reported by Kawamura *et al.*¹⁾ The compound 1 was crystallized from ethanol in spite of methanol by the method of Kushibe *et al.*,⁶⁾ giving a yield of 5.1 g. The compound 2 was further purified by gel-filtration using Toyopearl HW-40S column (φ50×950 mm, 1800 mL) and the lyophilized powder of compound 2 (1.9 g) was finally obtained.

The compounds 1 and 2 showed almost the same ¹H- and ¹³C-NMR spectra and ¹³C-NMR spectra of the two compounds were superimposed on those of cycloinulohexaose (CF6) previously reported.¹⁾ The positive FAB-MS spectrum of the compound 1 showed a protonated molecular ion peak at $m/z=973[M+H]^+$ and therefore was identified as CF6. In the case of compound 2, a protonated molecular ion peak at $m/z=1135[M+H]^+$ was obtained by the FAB-MS. Accordingly, the compound 2 was identified as cycloinulohexaose (CF7), and was also confirmed by elementary analysis (Calcd. for C₄₂H₇₀O₃₅·3H₂O: C, 42.43; H, 6.44. Found: C, 42.50; H, 6.73).

Analytical Methods. CFs were analyzed by a Waters HPLC system (Waters 2690) equipped with a Shodex RI-101 detector under two conditions as below: (A) column, MCI GEL CK04SS (φ10×200 mm, Mitsubishi Chemical Co.); mobile phase, distilled water; column temperature, 60°C; flow rate, 0.3 mL/min. (B) column, Shodex Asahi-Pak NH2P-504E (φ4.6×250 mm, Showa Denko K.K.); mobile phase, acetonitrile/water (3/1, v/v); column temperature, room temperature; flow rate, 0.8 mL/min. NMR spectra were recorded on a JEOL JNM-GSX500M system. Samples (10 mg) were dissolved in 0.5 mL of D₂O. FAB-MS analysis was carried out by a JEOL JMS DX-303 system. Elementary analysis was performed at Microanalytica Narita in Shizuoka, Japan.

CFTase activity. A reaction mixture (1 mL) containing 2% inulin (chicory roots) and an enzyme solution in 40 mM sodium phosphate buffer (pH 7.0) were incubated for 60 min at 40°C. The reaction was stopped by heating for 5 min in a boiling water bath. Then, CFs were determined by HPLC under the conditions A as stated above, with CF6 and CF7 as standards. One unit of CFTase activity was defined as the amount of enzyme that formed 1 micromole of CFs (CF6+CF7) per min.

Preparation of Cycloinulohexaose-Sepharose CL-6B.

Cyanogen bromide-activated Sepharose CL-6B (25 mL) prepared according to the method of March *et al.*¹⁵⁾ was suspended in 50 mL of 0.5 M 1,6-hexane diamine (pH 10.0) and shaken for 5 h at room temperature. The resulting aminohexyl(AH)-Sepharose was washed with 150 mL each of the following: 1 M NaHCO₃ containing 0.5 M NaCl, distilled water, 1 N HCl, distilled water, 1 N NaOH and finally distilled water. Next, cyanogen bromide-activated CF6 was prepared by reacting 50 mL of 1 M NaHCO₃ containing 2.5 g of CF6 with 5 mL of acetonitrile containing 2.5 g of cyanogen bromide for 3 min at 4°C. To the reaction mixture was added the AH-Sepharose and the suspension was shaken for 18 h at 4°C. The resultant gel was washed successively with 1 M NaHCO₃, 1 M KCl, 1 M sodium acetate and distilled water (250 mL, respectively). The CF6-Sepharose CL-6B thus prepared was stored in 0.05% sodium azide solution at 4°C until use.

Purification of CFTase. Unless otherwise indicated, all operations for the enzyme purification were carried out at 0–4°C.

Step 1: Ammonium sulfate fractionation. After cultivation of the microorganism, the cells were removed from the culture broth by centrifugation at 18,500×*g* for 20 min. Solid ammonium sulfate was added to the supernatant (780 mL) to obtain 80% saturation. After standing overnight, the precipitate was collected by centrifugation and then dissolved in 8.5 mL of 50 mM Tris-HCl buffer (pH 7.0). The solution was passed through a Sephadex G-25 column (φ26×550 mm) previously equilibrated with the same buffer. Elution patterns were monitored by a Pharmacia UV-2 monitor.

Step 2: DEAE-Toyopearl 650 M chromatography.

The eluate (92 mL) obtained at step 1 was applied to a column (φ25×180 mm) of DEAE-Toyopearl 650 M which had been equilibrated with 50 mM Tris-HCl buffer (pH 7.0). The column was washed with 450 mL of the above buffer and then eluted with a 1000 mL linear gradient of 0 to 0.25 M NaCl in the same Tris-HCl buffer at a flow rate of 115 mL/h and 17-mL fractions were collected. The fractions (216 mL) containing enzyme activity was collected and concentrated to 20 mL with a Centrplus YM-30 ultrafiltration membrane (Millipore Co.).

Step 3: Affinity chromatography on CF6-Sepharose CL-6B. The enzyme solution obtained from step 2 was applied to CF6-Sepharose CL-6B column (φ14×83 mm) previously equilibrated with 50 mM Tris-HCl buffer (pH 7.0). After washings with 80 mL of the same buffer, the enzyme was eluted with the buffer containing 0.5% inulin (chicory roots) at a flow rate of 20 mL/h and 2-mL fractions were collected. The fractions (32 mL) having enzyme activity was pooled and then concentrated to 1.4 mL by the ultrafiltration as mentioned in step 2.

Step 4: Gel-filtration on Superdex 200. The enzyme solution obtained from step 3 was applied to Superdex 200 prep column (φ16×760 mm) previously equilibrated with 50 mM Tris-HCl buffer (pH 7.0). The resulting effluent was re-circulated back onto the column at a flow rate of 20 mL/h and this re-circulation was repeated 5 times. Elution profile was monitored by the Pharmacia UV-2

monitor during the re-circulation. Then, the column was eluted with 50 mM Tris-HCl buffer (pH 7.0) and 1.5-mL fractions were collected. The fractions (14 mL) containing enzyme activity were pooled and concentrated to 1.3 mL by the ultrafiltration with a Centriplus YM-30.

Analytical methods for enzyme. Protein was determined by the method of Lowry *et al.*¹⁶⁾ with bovine serum albumin as a standard. Polyacrylamide gel electrophoresis was performed by a Phastsystem (Amersham Pharmacia Biotech Ltd.). The purity and relative molecular mass of the enzyme were examined by SDS-PAGE using 7.5% gel (PhastGel Homogeneous 7.5), with a HMW SDS Marker Kit (Amersham Pharmacia Biotech Ltd.) as molecular markers. Gels were stained with Coomassie Brilliant Blue G-250. Estimation of the molecular mass by gel-filtration was carried out by a Waters HPLC system (Waters 2690) equipped with TSK-GEL SUPER SW 3000 (ϕ 4.6×300 mm, Tosoh Co.). The column was eluted with 0.1 M sodium phosphate buffer (pH 7.0) containing 0.1 M sodium sulfate and 0.025% sodium azide at a flow rate of 0.3 mL/min at room temperature with a Gel Filtration Molecular Markers (Sigma), and the elution profile was monitored by a Waters UV detector (Waters 2487) at 280 nm. *N*-Terminal amino acid sequence of the enzyme was analyzed on a Shimadzu PPSQ-10 Protein Peptide Sequencer at Shizuoka University, Japan. Comparison of the sequence with other protein sequences was performed using a FASTA data base.

Nucleotide sequence of CFTase gene (*cft*). Chromosomal DNA was prepared from *P. polymyxa* MG-CF6 by the method of Saito and Miura.¹⁷⁾ A core sequence of *cft* was amplified from the DNA by polymerase chain reaction (PCR). Primers used were designed on the basis of the *N*-terminal amino acid sequence of the CFTase and the nucleotide sequences which are highly conserved regions of three CFTase gene previously reported.^{10,18,19)} Furthermore, to determine the upstream and the downstream sides of the core sequence inverse PCR was done. These nucleotides of resultant PCR products were sequenced with an Applied Biosystems 373A DNA sequencer and a DNA sequencing kit (ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit). The nucleotide sequence of the CFTase was assigned DDBJ accession no. AB 379660.

Cloning and expression of the CFTase gene from *P. polymyxa* MG-CF6 in *Escherichia coli*. A DNA fragment containing the CFTase gene was amplified by PCR with Ex Taq DNA polymerase (Takara Bio Inc., Kyoto, Japan) and with sense primer containing a *Eco*RI site, the Shine-Dalgarno (SD) sequence, and the ATG start codon (5'-GGGAATTCAGGAAACAGACCATGGATATCAAGCCGGATTTCAAGCG-3') and an antisense primer containing a *Pst*I site (5'-GGCTGCAGTCAGTTGTTGTTTCTTCTTTACCTGAACAGG-3'). The sense primer was designed on the basis of the *N*-terminal amino acid sequence of the CFTase from *P. polymyxa* MG-CF6. Furthermore, to obtain the CFTase containing *N*-terminal amino acid sequence corresponding to that of the CFTase from *Bacillus macerans* CFC1, a DNA fragment was amplified with sense primer containing a *Eco*RI site, the SD sequence, the ATG start codon (5'-GGGAATTCAGGAA

ACAGACCATGTTCCCCAAGCAAGTGGTAAGGGTAC-3') and the same antisense primer as mentioned above. Each of the two amplified DNA fragments (122 and 108 kDa enzymes) was ligated into the *Eco*RI-*Pst*I site of pUC18. The resultant plasmids, pCFTASE-122 and pCFTASE-108, were introduced into *E. coli* JM109 to provide recombinant 122 kDa and 108 kDa CFTase. *E. coli* JM109 carrying the each pCFTASE was cultured in LB medium containing 50 µg/mL ampicillin and 200 µg/mL isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C for overnight.

RESULTS

Identification of microorganism.

A strain MG-CF6 that produces CFs from inulin was classified as *Paenibacillus polymyxa* from taxonomic characteristics as shown in Table 1. DNA-DNA hybridization value between type strain of *Paenibacillus polymyxa* and the strain MG-CF6 was found to be 85%. In addition, comparative sequence analysis of 16S rDNA between the strain MG-CF6 and the type strain revealed that the strain MG-CF6 possessed 100% sequence similarity to the type strain. As a result, the strain was identified as *Paenibacillus polymyxa* MG-CF6.

Purification of CFTase.

A summary of the specific activity and recovery of the enzyme during the purification procedure based on 780 mL of the culture supernatant is given in Table 2. The enzyme was purified 500-fold with a yield of about 22%. In the purification procedure, affinity chromatography on the CF6-Sepharose was very effective for purification of the enzyme. The purified CFTase showed a single protein band on SDS-PAGE (Fig. 1). The molecular mass of the enzyme was estimated to be 128 kDa by SDS-PAGE and 118 kDa by gel filtration method. The isoelectric point of the enzyme was estimated to be around pH 5.1 and the enzyme was microheterogeneous (data not shown).

General properties.

General properties of the purified CFTase are summarized in Table 3. Optimum pH of the CFTase activity was measured in McIlvaine buffer (pH 3.0–8.0) and in 0.1 M borate-NaOH buffer (pH 8.0–10.0) in the presence of 2% inulin at 40°C. The enzyme showed a maximum activity at pH 7.0 and retained more than 80% of its maximum activity in the range of pH 6.0 to 8.0. The pH stability of the enzyme was examined by incubation of the enzyme for 18 h at 4°C in the respective buffers (ranging from pH 3.0 to 10.0) prior to assay at its optimum pH. The enzyme was stable in the range of pH 6.0 to 9.0. Optimum temperature of the enzyme was determined by measuring under the standard conditions and varying temperatures from 30 to 70°C. The enzyme showed its maximum activity at 45°C and more than 80% of its maximum activity was observed between 40 and 50°C. After incubation of the enzyme at different temperatures for 30 min, the enzyme activity was measured under standard conditions. The enzyme was stable up to 40°C and lost its activity at 60°C. The CFTase was completely inhibited by 1 mM Ag⁺ ion

Table 1. Taxonomic characteristics of *Paenibacillus polymyxa* MG-CF6.

Characteristics	Type strain	MG-CF6
Gram reaction	–	–
Motility	+	+
Length of rod (micro meter)	ND	2.8–3.5
Diameter of rod (micro meter)	ND	1.7–2.4
Spore position and shape	Center, ellipsoidal	Center, ellipsoidal
Sporangium swollen	+	NT
Growth at		
10°C	+	–
30°C	+	+
40°C	+	+
50°C	–	–
Catalase	+	+
Growth in the presence of		
2%NaCl	ND	+
5%NaCl	–	–
7%NaCl	–	–
10%NaCl	–	–
Anaerobic growth	+	+
Acid from		
Glucose	+	+
Cellobiose	+	+
Galactose	+	+
Mannose	+	+
Melibiose	+	+
Raffinose	+	+
Salicin	+	+
Xylose	+	+
Lactose	–	+
Starch	+	+
Inulin	+	+
L-Rhamnose	–	–
Sorbitol	–	–
L-Sorbose	–	–
Adonitol	–	–
Gas from glucose	+	+
Utilization of citrate	–	–
Urease	–	–
Production of indole	–	–
Voges-Proskauer test	+	+
Egg-yolk reaction	–	–
Nitrate reduced to nitrite	+	–
Tyrosine degradation	–	–
Gelatin hydrolysis	+	+
Starch hydrolysis	+	+
Oxidase	–	–
Lysozyme resistance	+	+
Major fatty acid	Anteiso-C15:0, C16:0	Anteiso-C15:0, C16:0
GC content (mol%)	44.3–45.6	48.05
Murein type	Meso-DAP direct	meso-DAP
Main menaquinone	MK-7	MK-7

Meso-DAP, meso-diaminopimelic acid; MK-7, menaquinone 7; ND, not data available; NT, not tested.

(Table 4). The enzyme produced a mixture of CF6 and CF7, which had a molar ratio of about 5:2. The CFTase activity were examined with inulins derived from different sources (Table 5). The CFTase showed the highest activity toward inulin from chicory roots and the CFTase activities on the other inulins remained more than 90%

Table 2. Purification of cyclinulooligosaccharide fructanotransferase from *Paenibacillus polymyxa* MG-CF6.

Steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture	430	120	0.28	100	1.0
(NH ₄) ₂ SO ₄ fraction	93	88	0.95	73	3.4
DEAE-Toyopearl	11	80	7.3	67	26
CF6-Sepharose	0.27	38	140	32	500
Superdex 200	0.18	26	140	22	500

Fig. 1. SDS-PAGE of purified enzyme.

Right line, purified enzyme; left line, protein molecular mass markers; myosin from rabbit muscle (220 kDa), α -2-macroglobulin from bovine plasma (170 kDa), β -galactosidase from *E. coli* (116 kDa), transferrin from human (76 kDa), glutamate dehydrogenase from bovine liver (53 kDa).

Table 3. Comparison of general properties of purified CFTase with other CFTase previously reported.

Characteristics	CFTase					
	<i>P. polymyxa</i> MG-CF6	<i>P. polymyxa</i> MGL21	<i>P. polymyxa</i> MGL21	<i>P. macerans</i> CFC1	<i>B. circulans</i> MCI-2554	<i>B. circulans</i> OKUMZ31B
Molecular mass (kDa)	128	148	108	110	115	132
Optimum pH	7.0 ^{a)}	9.0	7.0	7.5	7.5	7.5
pH Stability	6.0–9.0 ^{b)}	7.0–10.0	–	6.0–9.5	5.5–9.5	6.0–9.0
Optimum temperature (°C)	45 ^{c)}	50	40	45	45	45
Thermal stability (°C)	Below 40 ^{c)}	Below 50	–	Below 45	Below 45	Below 40
Inhibition of metal ion	Ag ⁺ ^{d)}	Ag ⁺ , Cu ²⁺	Ag ⁺	Ag ⁺ , Cu ²⁺	Fe ²⁺ , Cu ²⁺	Hg ²⁺
Production ratio of CF6: CF7	2.5:1	2.7:1 ^{e)}	2.7:1 ^{e)}	4.0:1	4.0:1	4.0:1

^{a)}Optimum pH of the purified enzyme was measured in McIlvaine buffer (pH 3.0–8.0) and in 0.1 M borate-NaOH buffer (pH 8.0–10.0) in the presence of 2% inulin. ^{b)}For measurement of pH stability, the enzyme was incubated in the above buffers for 18 h at 4°C and then the remaining activity was assayed in 0.2 M sodium phosphate buffer, pH 7.0 in the presence of 2% inulin. ^{c)}Optimum temperature was measured at different temperature of 30–70°C according to the assay method described in MATERIALS AND METHODS. For measurement of thermal stability, the enzyme was incubated at the different temperatures for 30 min and then the activity was assayed. ^{d)}For examining the effects of metal ions, the enzyme activity was measured under optimum conditions in the presence of 1 mM metal ions. ^{e)}The ratio is obtained with crude enzyme from *P. polymyxa* MGL21.¹⁰⁾

Table 4. Effects of metal ions on CFTase activity.

Metal ions	Relative activity (%)
None	100
Na ⁺	102
Ag ⁺	0
Ca ²⁺	107
Co ²⁺	102
Cu ²⁺	100
Fe ²⁺	96
Mg ²⁺	103
Zn ²⁺	100
Al ³⁺	82
Fe ³⁺	100
EDTA	94

The enzyme activity was assayed under optimum conditions in the presence of 1 mM metal ions.

compared with those on the chicory inulin.

N-Terminal amino acid sequence.

N-Terminal amino acid sequence was determined up to 14 residues and was compared with other CFTase reported so far (Table 6). The *N*-terminal sequence was fully consistent with the amino acid sequence of 253 to 266 from *Bacillus macerans* CFC1¹⁹ and *Paenibacillus polymyxa* MGL21¹⁰ enzymes and also possessed about 78.5% similarity to the amino acid sequence (235–248) of CFTase from *Bacillus circulans* MCI-2554.¹⁸

DNA sequence and deduced amino acid sequence.

The entire open reading frame of *cft* consists of 3999 nucleotides encoding a protein of 1333 amino acids with a predicted molecular mass of 149,461 Da, as shown in Fig. 2. *N*-Terminal amino acid sequence (DIKPDFKRYEIDNP) of the purified CFTase was found in amino acid residues 253–266 of the deduced amino acid sequence. The molecular mass of the purified enzyme is estimated to be 121,942 Da, accordingly. The deduced amino acid sequence of the CFTase from *P. polymyxa* MG-CF6 showed 83, 95 and 98% similarity to that of *Bacillus circulans* MCI-2554,¹⁸ *Paenibacillus* (*Bacillus*) *macerans* CFC1,¹⁹ and *Paenibacillus* (*Bacillus*) *polymyxa* MGL21,¹⁰ respectively, as illustrated in Fig. 3.

Enzyme assay of the recombinant CFTase.

Cultured *E. coli* cells expressing 122 and 108 kDa CFTase were disrupted by sonication and centrifuged. The CFTase activities in the resultant crude extract were 0.22 U/mg for 122 kDa enzyme and 0.07 U/mg for 108 kDa enzyme, whereas the crude extract of *E. coli* JM109 carrying pUC18 was inert.

DISCUSSION

There have been only four reports concerning the purification and characterization of CFTase^{2,7,9,10} up until now. We purified and characterized the CFTase from *Paenibacillus polymyxa* MG-CF6 and its general properties are summarized in comparison with the other five CFTases (Table 3). Optimal PH (7.0) of the enzyme was different from that (pH 7.5) of the enzymes from *B. macerans*

Table 5. Effects of inulins from different sources on CFTase activity.

Inulin from	Relative activity (%)
Chycory roots	100
Jerusalem artichokes	99
Dahlia tuber	92
Rafterin HP	95
Fuji FF	94

The enzyme activity was measured under optimum conditions in the presence of 2% each inulin.

Table 6. Comparison of *N*-terminal amino acid sequences from the purified CFTase with peptides of CFTase from other origin.

CFTase from	Amino acid sequence
<i>P. polymyxa</i> MG-CF6	DIKPDFKRYEIDNP (<i>N</i> -terminal)
<i>P. polymyxa</i> MGL21	DIKPDFKRYEIDNP (253–266)
<i>B. macerans</i> CFC1	DIKPDFKRYEIDNP (253–266)
<i>B. circulans</i> MCI-2554	DIKPDFERYQIENP (235–248)

CFC1, *B. circulans* OKUMZ31B and *B. circulans* MCI-2554. The optimal pH of the enzyme was also distinct from that (pH 9.0) of the recombinant CFTase (148 kDa) from *P. polymyxa* MGL21,¹⁰ but was the same as that of recombinant CFTase (108 kDa) from the strain MGL21.¹¹ The enzyme was hardly inhibited by Cu²⁺ ion, which inhibited both the enzymes from *B. circulans* MCI-2554⁷ and *B. macerans* CFC1.⁹ With respect to the enzymes from *P. polymyxa* MGL21, the 148 kDa CFTase is reported to be inhibited by Cu²⁺ ion¹⁰ whereas no inhibition was observed either in the 108 kDa CFTase¹¹ or the enzyme in this study. The most remarkable difference between this enzyme and the enzymes from *B. circulans* OKUMZ31B,² *B. circulans* MCI-2554⁷ and *B. macerans* CFC1⁹ was the production ratio of cycloinulohexaose (CF6) and cycloinulohptaose (CF7) as shown in Table 3, indicating that the enzyme from *P. polymyxa* MG-CF6 is capable of producing larger amounts of CF7 than the other enzymes. In this connection, crude enzyme prepared from culture broth of *P. polymyxa* MGL21 was found to produce CF6 and CF7 in a ratio of 2.7:1,¹⁰ which was similar to that (2.5:1) in our purified enzyme.

N-Terminal amino acid sequence (DIKPDFK, amino acid position 253–259) of the purified enzyme was almost the same as that (TDIKPDFK, amino acid position 252–259) of 137 kDa CFTase from *P. polymyxa* MGL21,¹¹ in which one more amino acid residue (Thr) is attached to the *N*-terminal amino acid of our purified enzyme. Unfortunately, You *et al.*¹¹ have reported that the 137 kDa CFTase could not be purified because the strain MGL21 produced three CFTase with different molecular mass (148, 137 and 108 kDa) and these enzymes could not be separated in any purification processes. However, taking into account the similarity of the 137 kDa enzyme to our purified enzyme, it is considered that general properties of the 137 kDa enzyme are highly analogous to those of our enzyme. In addition, since general properties of a recombinant 108 kDa CFTase,¹¹ which was constructed by deleting a part of *N*-terminal polypeptide chain in the 137 kDa enzyme, were also very similar to those of our purified enzyme, *N*-terminal region of CFTase was thought

Fig. 2. Nucleotide sequence of CFTase gene and deduced amino acid sequence.

quence of our enzyme had 98% and 95% similarity to that of the MGL21 and the *B. macerans* CFTases, respectively, and accordingly was regarded as being made up of the five distinct regions. On the other hand, in spite of high similarity of the amino acid sequence among the three CFTases as mentioned above, significant differences in the production ratio of CF6 and CF7 were observed as

Pp6	HRKAK-GIKQWISGMVIMAWVLQVVGISVASBNRTVAGETLVFKNDVKSVSVDALVQIRNPKNDGLSVTDSVYQIRNPQFETGDLTGWTWVNGEAFQPSVSTBTWMAKISYNG	119
Pp21	HRKAK-GRKQWISGMVIMAWVLQVVGISVASBNRTVAGETLVFKNDVKSVSVDALVQIRNPKNDGLSVTDSVYQIRNPQFETGDLTGWTWVNGEAFQPSVSTBTWMAKISYNG	119
Bm	HRKVK-RGKQWISGMVIMAWVLQVVGISVASBNRTVAGETLVFKNDVKSVSVDALVQIRNPKNDGLSVTDSVYQIRNPQFETGDLTGWTWVNGEAFQPSVSTBTWMAKISYNG	119
Bc	HRKVKRGKKGFAALATWBLFFQLIAPGISAAPPSADIEDALISEASESEIPTYDAVYQIE-----NPQFETGDLTGWVNGEAFQPSVSTBTWMAKISYNG	101
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Pp6	EGAYHLNGNKYDESATGSIHSESTFELGGSGWISFRLGGAKNPKVFINIVEIDTQGVYARYGNSAFADVGFDFPALGMRLANHVCYKADLSTYLAKKLIVVEDNATSDMGVIFADAFSH	239
Pp21	EGAYHLNGNKYDESATGSIHSESTFELGGSGWISFRLGGAKNPKVFINIVEIDTQGVYARYGNSAFADVGFDFPALGMRLANHVCYKADLSTYLAKKLIVVEDNATSDMGVIFADAFSH	239
Bm	EGAYHLNGNKYDESATGVLRSSTFELGGSGGISFRLGGAKNPKVFINIVEIDTQGVYARYGNSAFADVGFDFPALGMRLANHVCYKADLSTYLAKKLIVVEDNATSDMGVIFADAFSH	239
Bc	EGAYHLNGNKYDESATGVLRSSTFELGGSGWISFRLGGAKNPKVFINIVEIDTQGVYARYGNSAFADVGFDFPALGMRLANHVCYKADLSTYLAKKLIVVEDNATSDMGVIFADAFSH	221

Pp6	YHESEPAEGIVATDIKFPDFKRSEIDNPSFET-----	270
Pp21	YHESEPAEGIVATDIKFPDFKRSEIDNPSFET-----	270
Bm	YHESEPAEGIVATDIKFPDFKRSEIDNPSFET-----	270
Bc	YHESEPAEGIVATDIKFPDFKRSEIDNPSFET-----	341

Pp6	-----GNLDGWTVEGDAPFVTEBAHASKEGNFF	298
Pp21	-----GNLDGWTVEGDAPFVTEBAHASKEGNFF	298
Bm	-----GNLDGWTVEGDAPFVTEBAHASKEGNFF	298
Bc	NLIARVCHSAPTDVGFDFPALGMRLANHVCYKADLSTYLAKKLIVVEDNATSDMGVIFADAFSH	461

Pp6	AKSSMQCKGISTENTFTLQCTQITNFTILDLKPERAYVALVDANSENTLLNKTCHNVANENKISWKVQEHYNNKLYVYVDSHNSIASVDSFQASCKCTIFELNLDGAGCKKALEKVNHL	418
Pp21	AKSSMQCKGISTENTFTLQCTQITNFTILDLKPERAYVALVDANSENTLLNKTCHNVANENKISWKVQEHYNNKLYVYVDSHNSIASVDSFQASCKCTIFELNLDGAGCKKALEKVNHL	418
Bm	AKSSMQCKGISTENTFTLQCTQITNFTILDLKPERAYVALVDANSENTLLNKTCHNVANENKISWKVQEHYNNKLYVYVDSHNSIASVDSFQASCKCTIFELNLDGAGCKKALEKVNHL	418
Bc	AKSSMQCKGISTENTFTLQCTQITNFTILDLKPERAYVALVDANSENTLLNKTCHNVANENKISWKVQEHYNNKLYVYVDSHNSIASVDSFQASCKCTIFELNLDGAGCKKALEKVNHL	581
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Pp6	RBDVNYVFNARYHASKDPFRWTFRGGALLPDGYSNHYEVNANDTVFVSDALTEIANVAFRSYEMGDGNKLSAIVNQSDQKABQFSLGMYRHSWTNMGVIGSSQNIQVWVNDHFLER	538
Pp21	RBDVNYVFNARYHASKDPFRWTFRGGALLPDGYSNHYEVNANDTVFVSDALTEIANVAFRSYEMGDGNKLSAIVNQSDQKABQFSLGMYRHSWTNMGVIGSSQNIQVWVNDHFLER	538
Bm	RBDVNYVFNARYHASKDPFRWTFRGGALLPDGYSNHYEVNANDTVFVSDALTEIANVAFRSYEMGDGNKLSAIVNQSDQKABQFSLGMYRHSWTNMGVIGSSQNIQVWVNDHFLER	538
Bc	RBDVNYVFNARYHASKDPFRWTFRGGALLPDGYSNHYEVNANDTVFVSDALTEIANVAFRSYEMGDGNKLSAIVNQSDQKABQFSLGMYRHSWTNMGVIGSSQNIQVWVNDHFLER	761

Pp6	YKNNYVAATFSEKDGKILKILNGBEASQATPVNVPITFSTESLIIGKNNKPYELAGVFSFNMPSGLLDEVLHNLKALTWQELLAGYESVVALHGGSSIFKIPNADIDEDPSVFDGQHRP	658
Pp21	YKNNYVAATFSEKDGKILKILNGBEASQATPVNVPITFSTESLIIGKNNKPYELAGVFSFNMPSGLLDEVLHNLKALTWQELLAGYESVVALHGGSSIFKIPNADIDEDPSVFDGQHRP	658
Bm	YKNNYVAATFSEKDGKILKILNGBEASQATPVNVPITFSTESLIIGKNNKPYELAGVFSFNMPSGLLDEVLHNLKALTWQELLAGYESVVALHGGSSIFKIPNADIDEDPSVFDGQHRP	658
Bc	YKNNYVAATFSEKDGKILKILNGBEASQATPVNVPITFSTESLIIGKNNKPYELAGVFSFNMPSGLLDEVLHNLKALTWQELLAGYESVVALHGGSSIFKIPNADIDEDPSVFDGQHRP	821

Pp6	QYHAMPQNNMMEAHAFIYNGXHYLFYQHNFGQFPHQIHGHWGWSDDMVNENVRPALAPFAGTLDPDGTWSSGAATDRNGNPLVFTTAGNDLSLSPNORTGLATPADLSDFYLEMKVK	778
Pp21	QYHAMPQNNMMEAHAFIYNGXHYLFYQHNFGQFPHQIHGHWGWSDDMVNENVRPALAPFAGTLDPDGTWSSGAATDRNGNPLVFTTAGNDLSLSPNORTGLATPADLSDFYLEMKVK	778
Bm	QYHAMPQNNMMEAHAFIYNGXHYLFYQHNFGQFPHQIHGHWGWSDDMVNENVRPALAPFAGTLDPDGTWSSGAATDRNGNPLVFTTAGNDLSLSPNORTGLATPADLSDFYLEMKVK	778
Bc	QYHAMPQNNMMEAHAFIYNGXHYLFYQHNFGQFPHQIHGHWGWSDDMVNENVRPALAPFAGTLDPDGTWSSGAATDRNGNPLVFTTAGNDLSLSPNORTGLATPADLSDFYLEMKVK	941

Pp6	YKKEVTEQNGKGIHNEFRDPPVWYDKEVDKQVQLVTSGLDPSSGTALVYVSDNNYHMYKGLPYVSDRIYVPELSTVWELPVLLPLGDSGTGKQYIFNINPEKPERHVPANDVQRDV	898
Pp21	YKKEVTEQNGKGIHNEFRDPPVWYDKEVDKQVQLVTSGLDPSSGTALVYVSDNNYHMYKGLPYVSDRIYVPELSTVWELPVLLPLGDSGTGKQYIFNINPEKPERHVPANDVQRDV	898
Bm	YKKEVTEQNGKGIHNEFRDPPVWYDKEVDKQVQLVTSGLDPSSGTALVYVSDNNYHMYKGLPYVSDRIYVPELSTVWELPVLLPLGDSGTGKQYIFNINPEKPERHVPANDVQRDV	898
Bc	YKKEVTEQNGKGIHNEFRDPPVWYDKEVDKQVQLVTSGLDPSSGTALVYVSDNNYHMYKGLPYVSDRIYVPELSTVWELPVLLPLGDSGTGKQYIFNINPEKPERHVPANDVQRDV	1061

Pp6	EVFVMIQVNDNRNFKFIPDQKASSKNDVCDGYFTAESGLVTPDGRVTFVFNQVNRTPQAEYQSGWANHLSLPVLSLDKSLKHLIEPIKELQTLRCKKWDVFSKKNLEBANQILINKVK	1018
Pp21	EVFVMIQVNDNRNFKFIPDQKASSKNDVCDGYFTAESGLVTPDGRVTFVFNQVNRTPQAEYQSGWANHLSLPVLSLDKSLKHLIEPIKELQTLRCKKWDVFSKKNLEBANQILINKVK	1018
Bm	EVFVMIQVNDNRNFKFIPDQKASSKNDVCDGYFTAESGLVTPDGRVTFVFNQVNRTPQAEYQSGWANHLSLPVLSLDKSLKHLIEPIKELQTLRCKKWDVFSKKNLEBANQILINKVK	1018
Bc	EVFVMIQVNDNRNFKFIPDQKASSKNDVCDGYFTAESGLVTPDGRVTFVFNQVNRTPQAEYQSGWANHLSLPVLSLDKSLKHLIEPIKELQTLRCKKWDVFSKKNLEBANQILINKVK	1181
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Pp6	DILEIVMEIDPREAQKPGKLVRSSEKQEBTLIYYDKKNGTFNVDRTKSSIDPVRVDIGQGYVLDLGENLKLHIFLDASVIEAFANYKKLITRVYVGRYDGLQINWAGDQINIKSH	1138
Pp21	DILEIVMEIDPREAQKPGKLVRSSEKQEBTLIYYDKKNGTFNVDRTKSSIDPVRVDIGQGYVLDLGENLKLHIFLDASVIEAFANYKKLITRVYVGRYDGLQINWAGDQINIKSH	1138
Bm	DILEIVMEIDPREAQKPGKLVRSSEKQEBTLIYYDKKNGTFNVDRTKSSIDPVRVDIGQGYVLDLGENLKLHIFLDASVIEAFANYKKLITRVYVGRYDGLQINWAGDQINIKSH	1138
Bc	DILEIVMEIDPREAQKPGKLVRSSEKQEBTLIYYDKKNGTFNVDRTKSSIDPVRVDIGQGYVLDLGENLKLHIFLDASVIEAFANYKKLITRVYVGRYDGLQINWAGDQINIKSH	1301

Pp6	QVWDMHALTGKFAAPVYVPRKMDNSVYKIDITELPNHDFATGDLTGWITEGDAFQNVHVTNQFFMDNIYFNESHKIPGGYHLNGFNBQAGGDSLTGALRSQNFVLGNGRINFLISGGRD	1258
Pp21	QVWDMHALTGKFAAPVYVPRKMDNSVYKIDITELPNHDFATGDLTGWITEGDAFQNVHVTNQFFMDNIYFNESHKIPGGYHLNGFNBQAGGDSLTGALRSQNFVLGNGRINFLISGGRD	1258
Bm	QVWDMHALTGKFAAPVYVPRKMDNSVYKIDITELPNHDFATGDLTGWITEGDAFQNVHVTNQFFMDNIYFNESHKIPGGYHLNGFNBQAGGDSLTGALRSQNFVLGNGRINFLISGGRD	1258
Bc	QVWDMHALTGKFAAPVYVPRKMDNSVYKIDITELPNHDFATGDLTGWITEGDAFQNVHVTNQFFMDNIYFNESHKIPGGYHLNGFNBQAGGDSLTGALRSQNFVLGNGRINFLISGGRD	1421

Pp6	IDRLYVALVRASDGKELFKETATNYEYQKIDWASDYIGEBLYIKVVCQSGKGGFGBINVDQFNVPVQVKKKTNH-----	1333
Pp21	IDRLYVALVRASDGKELFKETATNYEYQKIDWASDYIGEBLYIKVVCQSGKGGFGBINVDQFNVPVQVKKKTNH-----	1333
Bm	IDRLYVALVRASDGKELFKETATNYEYQKIDWASDYIGEBLYIKVVCQSGKGGFGBINVDQFNVPVQVKKKTNH-----	1333
Bc	IDRLYVALVRASDGKELFKETATNYEYQKIDWASDYIGEBLYIKVVCQSGKGGFGBINVDQFNVPVQVKKKTNH-----	1503

Fig. 3. Alignment of amino acid sequence of CFTase.

Pp6, CFTase from *P. polymyxa* MG-CF6 (accession no. AB379660); Pp21, CFTase from *P. polymyxa* MGL21 (accession no. AY077612); Bm, CFTase from *B. macerans* CFC1 (accession no. AF222787); Bc, CFTase from *B. circulans* MCI-2554 (accession no. D87672). The asterisk indicates residues identical among the four sequences. The *N*-terminal amino acid sequences shown in Table 6 are boxed. The residue numbers of amino acids in each line are shown on the right.

indicated in Table 3. Namely, the production ratio of our purified enzyme was clearly distinct from that of the *B. macerans* CFTase, but was almost the same as the MGL 21 one. You *et al.*¹¹⁾ have reported that *C*-terminal repeating region deleted enzymes of *P. polymyxa* MGL21 lose both CFTase and CF hydrolyzing activities while main-

taining inulin hydrolyzing activity and thus, the substrate binding site for CF6 and CF7 formation exists in the *C*-terminal repeating region. Accordingly, we compared amino acid sequences in the *C*-terminal repeating region of the three enzymes. There was no difference in the sequences of the *C*-terminal region between the MG-CF6

and MGL21 CFTases, but on the other hand, a relatively large difference (about 7%) in the sequences of the C-terminal region was found as compared with the MG-CF6 and the *P. macerans* enzymes. From these findings, it may be reasonably supposed that the difference in the production ratio of CF6 and CF7 between our enzyme and the *P. macerans* enzyme is because of the different primary structures in the C-terminal polypeptide chain.

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Paenibacillus polymyxa MG-CF6 の生産する シクロイヌロオリゴ糖生成酵素の精製と諸性質

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シクロイヌロオリゴ糖生成酵素を生産する土壌細菌を単離し *Paenibacillus polymyxa* MG-CF6 と同定した。本菌株が生成するシクロイヌロオリゴ糖生成酵素を培養上清液から SDS 電気泳動で単一バンドになるまで精製した。本酵素の分子質量は 128kDa, 至適 pH7.0, 至適温度 40°C であった。また、本酵素の pH 安定性は 6.0–9.0, 温度安定性は 40°C 以下であった。本酵素は、シクロイヌロヘキサオースとシクロイヌロヘプタオースを 2.5:1 の比率で生成した。本酵素の N-末端アミノ酸配列は、*Bacillus macerans* CFC1 および *Bacillus polymyxa* MGL21 の生産するシクロイヌロオリゴ糖生成酵素のアミノ酸配列の一部分と完全に一致した。さらに、本酵素の遺伝子配列を解析したところ、本酵素は 3999 のヌクレオチド配列にコードされた 1333 残基のアミノ酸からなるタンパク質であることが明らかになった。本酵素のアミノ酸配列を既知の酵素のアミノ酸配列と比較したところ、本酵素は *Bacillus circulans* MCI-2554, *Bacillus macerans* CFC1 および *Bacillus polymyxa* MGL21 の生産するシクロイヌロオリゴ糖生成酵素のアミノ酸配列とそれぞれ 83, 95 および 98% の相同性を有することがわかった。