J. Microbiol. Biotechnol. (1998), **8**(3), 251 – 257

JOURNAL OF
MICROBIOLOGY AND
BIOTECHNOLOGY

Purification and Characterization of Cycloinulooligosaccharide Fructanotransferase from *Bacillus macerans* CFC1

KIM, HWA-YOUNG AND YONG-JIN CHOI*

Graduate School of Biotechnology, Korea University, Seoul 136-701, Korea

Received: February 12, 1998

Abstract Cycloinulooligosaccharide fructanotransferase (CFTase) which produces cyclofructan from inulin was purified 332-fold from a culture broth of *Bacillus macerans* CFC1. The molecular mass of the CFTase was estimated to be 110 kDa by SDS-polyacrylamide gel electrophoresis and gel filtration, indicating that the enzyme has a monomer structure. The maximal level of enzyme activity was observed at pH 7.5 and 45°C. The enzyme was stable in the pH range 6.0 to 9.5, and at temperatures up to 45°C for 1 h. The enzyme activity was completely inhibited in the presence of 0.5 mM Ag^+ or Cu^{2+} ion. None of sucrose (GF), 1-kestose (GF2), or nystose (GF3) were found to be substrates for the CFTase, but inulooligosaccharides larger than nystose were attacked by the enzyme. The CFTase catalyzes not only the cyclization as the major reaction, but also disproportionation and coupling reactions involving intermolecular transfructosylation in the same manner as cyclodextrin glucanotransferase (CGTase) (EC 2.4.1.19).

Key words: Cycloinulooligosaccharide fructanotransferase, CFTase, *Bacillus macerans*, transfructosylation, purification

Inulin is a polyfructan consisting of a linear β-(2→1)-linked polyfructose chain having a terminal glucose residue. It is found as a reserve carbohydrate in various plants such as chicory, dahlia, and jerusalem artichoke. Inulin can be converted into fructose, inulooligosaccharides, cycloinulooligosaccharides, and difructofuranose anhydrides (DFAs) by various types of microbial inulin-decomposing enzymes. Inulin is thus of growing interest as a renewable carbohydrate raw material for sugar, food, fuel, and other industries. The inulin-decomposing enzymes reported so far are 2,1-β-D-fructan fructanohydrolase (EC 3.2.1.7) (inulinase) [1], inulin fructotransferase (EC 2.4.1.93) (inulinase II) [13, 14, 17], inulin fructotransferase (DFA I-producing) (EC 2.4.1.200) [2], and cycloinulooligosaccharide fructanotransferase (CFTase) [4, 7, 11].

CFTase converts inulin into cycloinulooligosaccharides (cyclofructans) of β-(2→1)-linked cyclic D-fructofuranose. Cyclofructan has a characteristic crown ether in the central part of the molecule and can bind cationic molecules via charge-dipole electrostatic interactions [16]. Kawamura and Uchiyama reported that CFTase from *Bacillus circulans* OKUMZ31B [6] produces mainly cycloinulohexaose (CF6) and a small amount of cycloinuloheptaose (CF7) and cycloinulooctaose (CF8) from inulin by catalyzing the intramolecular transfructosylation. This enzyme was also shown to catalyze coupling and disproportionation reactions involving intermolecular transfructosylations in the same manner as cyclodextrin glucanotransferase (CGTase) (EC 2.4.1.19). The CGTase catalyzes α-(1→4) transglycosylation to produce cyclodextrin from α-(1→4) glucan [5].

In our previous study [7], we isolated from soil a bacterial strain, *Bacillus* sp. CFC1, which produced an extracellular CFTase. This enzyme produced CF6 as a major product from inulin. By using the VITEX identification system, the CFTase producer could be identified as a strain of *Bacillus macerans*. In this study, we purified and characterized the CFTase from *Bacillus macerans* CFC1. In addition, we also investigated the transfructosylation reactions as well as cyclization reaction catalyzed by the CFTase.

MATERIALS AND METHODS

Bacterial Strain and Cultivation Conditions

Bacillus macerans CFC1 isolated from soil was used in this study. The bacterial strain was cultivated in the optimal medium established for the production of CFTase [7] at 37°C for 48 h on a rotary shaker (200 rpm).

*Corresponding author
Phone: 82-2-3290-3417; Fax: 82-2-923-9923;
E-mail: choiyj@kuccnx.korea.ac.kr

Enzyme Assay

The assay mixture for CFTase activity consisted of 0.5 ml of 5% (w/v) inulin in 50 mM phosphate buffer (pH 7.0) and 0.05~0.5 ml of enzyme solution. The reaction mixture was adjusted to a total volume of 1.0 ml by the addition of 50 mM phosphate buffer (pH 7.0). The mixture was incubated at 37°C for 1 h, and the reaction was stopped by heating the mixture in boiling water for 10 min. The reaction product, CF6, was determined by HPLC. Conditions for HPLC analysis were as follows: column, High Performance Carbohydrate column (4.6×250 mm, Waters, U.S.A.); detector, RI detector (Differential Refractometer R401, Waters, U.S.A.); mobile phase, acetonitrile:water (70:30); flow rate, 1.5 ml/min; and column temperature, 70°C. One unit of the enzyme activity was defined as the amount of enzyme producing 1 μmole of CF6 per min under these assay conditions.

Purification of CFTase

Culture broth was centrifuged with a Beckman JA10 rotor at 5,000 rpm for 15 min. The supernatant obtained was used as a crude enzyme solution. It was then fractionated with ammonium sulfate at 60~80% saturation. The fractionated proteins were centrifuged at 7,000 rpm at 4°C for 40 min. The precipitate was redissolved in 20 ml of 50 mM sodium phosphate buffer (pH 7.0) and dialyzed at 4°C overnight against the same buffer. The dialyzed enzyme solution was loaded on a DEAE-Sepharose CL-6B column (2.6×20 cm) preequilibrated with the same buffer. The adsorbed proteins were eluted with a linear gradient of 0 to 0.5 M NaCl in the same buffer. The fractions containing CFTase activity were gathered and concentrated by ultrafiltration, and dialyzed against the same buffer. The concentrated protein solution was loaded on a Sephacryl S-200 column (1.6× 75 cm) preequilibrated with the same phosphate buffer and then eluted with the same buffer at a flow rate of 20 ml/h. The fractions showing CFTase activity were collected and concentrated by Centriplus™-10 (Amicon, Co., U.S.A., 3,000×g, 45 min). The concentrated enzyme solution was run on a Superdex HR 200 column by FPLC (Pharmacia, Co., U.S.A.). The column buffer was 50 mM sodium phosphate buffer (pH 7.0) and the flow rate was 0.2 ml/min.

Gel Electrophoresis and Zymogram

SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli [12]. The gel was stained with Coomassie Brilliant Blue R250.

For the zymogram, the enzyme solution was electrophoresed on a SDS-polyacrylamide gel containing incompletely solublized 2% (w/v) inulin. The electrophoresed gel was washed four times for 30 min with 50 mM phosphate buffer of pH 7.0 (the first two washes contained 25% (v/v) isopropanol). The washed gels were then incubated in the same buffer for 2 h at 35°C. The activity bands appeared as transparent bands on the gel.

Intermolecular Transfructosylation Reactions

In order to test the coupling reaction, we used sucrose, 1-kestose (GF2), and nystose (GF3) as fructosyl acceptors, and CF6 contaminated with a small amount of CF7 as a fructosyl donor. For the disproportionation reaction, 1-β-fructofuranosyl nystose (GF4) was used as a fructosyl acceptor and also as a donor molecule. The reaction mixture consisted of 40 μl of 2% acceptor molecule, 40 μl of 2% donor, and 20 μl (100 mU) of the purified CFTase. The coupling and disproportionation reactions were carried out at 37°C for 16 h and 6 h, respectively. The reaction products were analyzed by HPLC as described above. The standard GF2, GF3, and GF4 were purchased from Meiji Pharmaceutical Co. (Japan) and CF6 purified in our labaratory as described previously [7] was used.

RESULTS

Purification of Enzyme

CFTase was obtained as an extracellular enzyme in the culture broth of *Bacillus macerans* CFC1. Table 1 summarizes typical results of the four-step purification. In the second step of anion-exchange chromatography on the DEAE-Sepharose CL-6B column, the enzyme was effectively separated from other proteins in the enzyme solution obtained from the ammonuim sulfate fractionation. The purified CFTase gave a single protein band on SDS-polyacrylamide gel as shown in Fig. 1A. The enzyme was purified 332-fold with a yield of 12.1%.

Table 1. Purification of CFTase from *Bacillus macerans* CFC1.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Culture supernatant	1402	68.6	0.05	100	1
Ammonium sulfate fractionation	132	35.4	0.27	51.6	5.4
DEAE-Sepharose CL 6B	7.8	22.5	2.88	32.8	57.6
Sephacryl S-200	1.2	13.3	11.08	19.4	221.6
Superdex HR 200	0.5	8.3	16.60	12.1	332.0

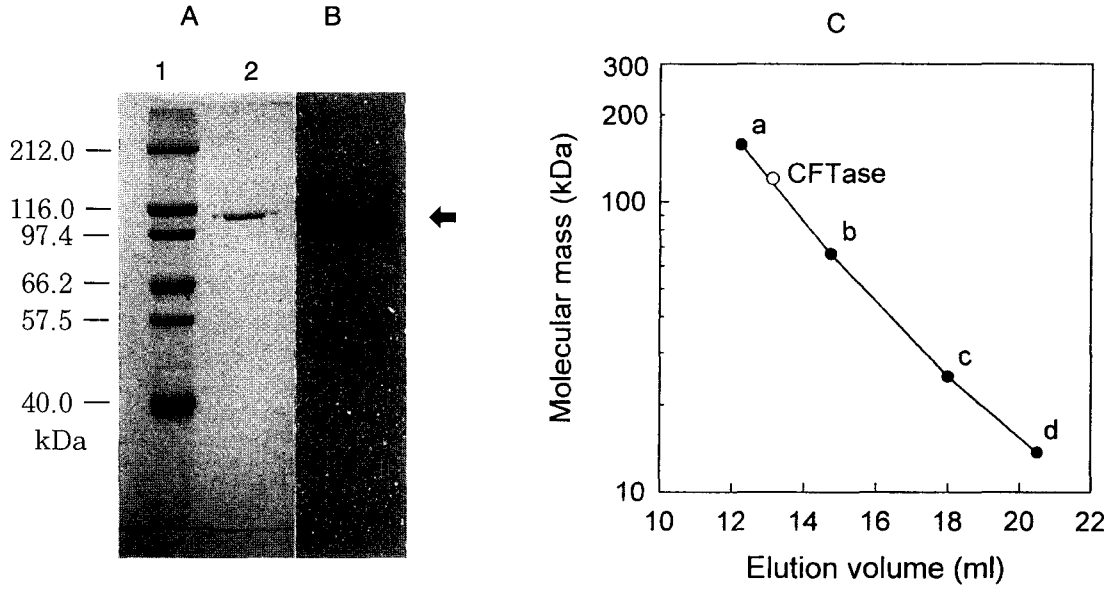

Fig. 1. Estimation of molecular mass of the purified CFTase from *Bacillus macerans* CFC1.
A. 8% SDS-polyacrylamide gel electrophoresis. Lane 1, marker proteins; lane 2, purified enzyme. B. Zymogram of purified enzyme on 8% SDS-polyacrylamide gel. Arrow indicates the purified CFTase. C. Molecular mass of the enzyme was determined by gel filtration chromatography with Superdex HR 200. a, aldolase (158 kDa); b, bovine serum albumin (66 kDa); c, chymotrypsinogen A (25 kDa); d, ribonuclease A (13.7 kDa).

Estimation of Molecular Mass

The molecular mass of the CFTase was estimated to be about 110 kDa by SDS-polyacrylamide gel electrophoresis (Fig. 1A). By gel filtration with Superdex HR 200, the molecular mass was determined to be approximately 110 kDa (Fig. 1C). From zymogram analysis, a single band of the purified CFTase could be detected on the SDS-polyacrylamide gel (Fig. 1B). These results indicate that the functional CFTase of *Bacillus macerans* CFC1 has a monomeric structure.

General Properties

Effects of pH and temperature on the enzyme activity
The enzyme activity was measured in the pH range 4.0 to 9.5. As shown in Fig. 2A, maximal activity was

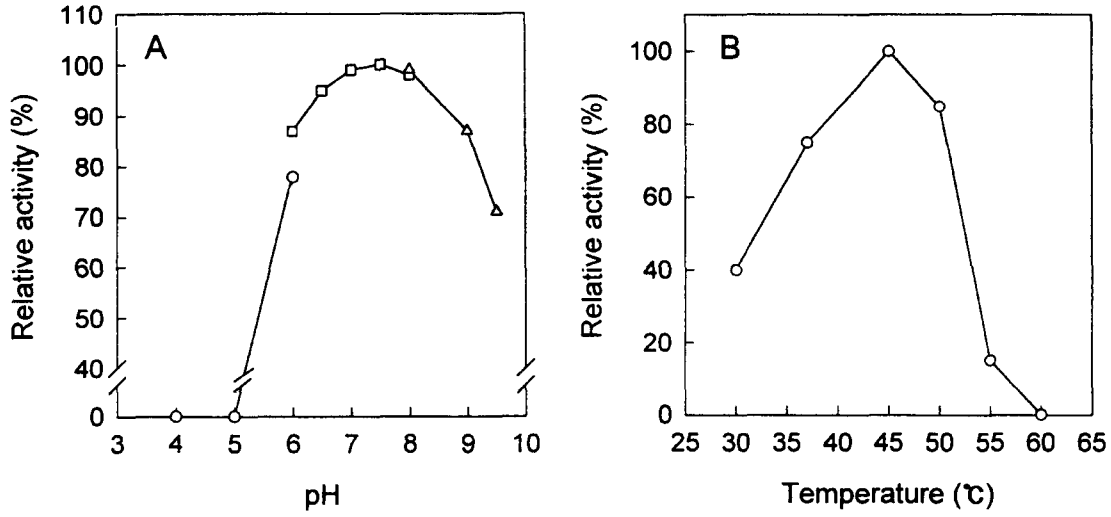

Fig. 2. Effects of pH (A) and temperature (B) on the enzyme activity.
A. Enzyme activity was measured in pH range 4.0 to 9.5 (○, 50 mM acetate buffer; □, 50 mM phosphate buffer; △, 50 mM Tris-Cl buffer). Enzyme reaction was carried out at 37°C for 1 h. Relative activity represents the relative value to the maximum enzyme activity at pH 7.5. B. Enzyme reaction solution in 50 mM phosphate buffer, pH 7.0, was incubated for 1 h at various temperatures. Relative activity was determined to be relative value to the activity at 45°C.

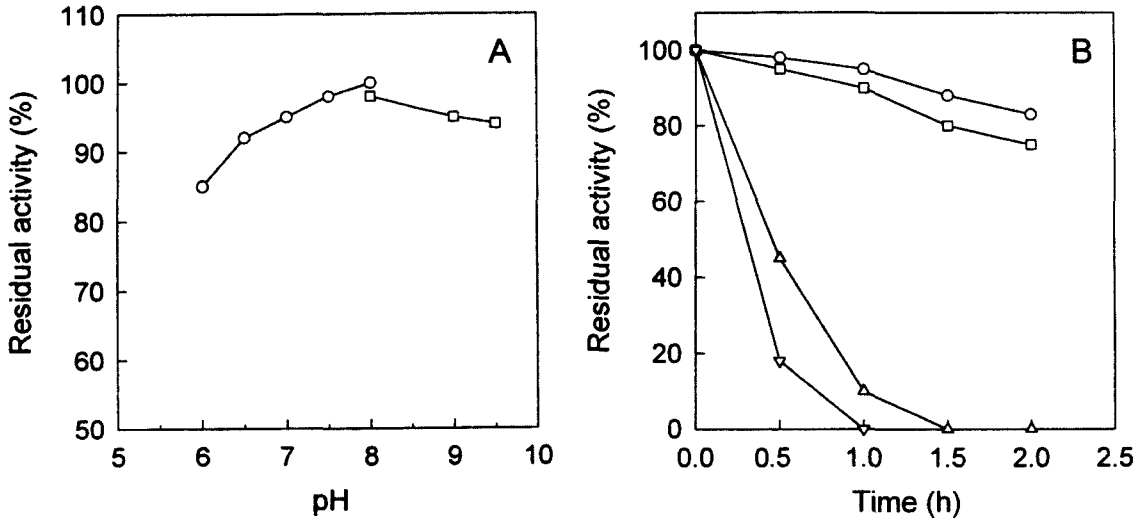

Fig. 3. pH (A) and thermal (B) stability.
A. Enzyme solution was incubated at 4°C for 18 h in 50 mM phosphate buffer (○, pH 6.0-8.0) or 50 mM Tris-Cl buffer (□, pH 8.0-9.5). After incubation, enzyme reaction was carried out at 45°C for 1 h and then the residual activity was measured. B. Residual enzyme activity was measured at 45°C for 30 min allowing the enzyme solution to stand at various temperatures (○, 40°C; □, 45°C; △, 50°C; ▽, 60°C) in 30 min intervals.

observed at pH 7.5. The effect of temperature on the enzyme activity was determined in the range of 30°C to 60°C. The optimal temperature was observed to be 45°C (Fig. 2B).

pH and thermal stability The purified enzyme was incubated at 4°C for 18 h in each of the buffers shown in Fig. 3A (pH range of 6.0 to 9.5). As seen in the figure, more than 85% of the enzyme activity remained in pH range 6.0 to 9.5. The enzyme was found to be stable up to 45°C for 1 h as shown in Fig. 3B.

Effect of metal ions on the enzyme activity CFTase activity was measured in the presence of various metal ions (Table 2). The enzyme activity was increased by about 10~20% in the presence of 1 mM Mg^{2+}, Fe^{2+}, Fe^{3+}, or Co^{2+}. On the contrary, Cu^{2+} and Ag^{+} ions inhibited the CFTase activity completely even at a concentration of 0.5 mM.

Table 2. Effect of metal ions on CFTase activity.

Metal ions	Relative activity (%)	
	1 mM	0.5 mM
None	100	100
Ag^{+}	0	0
Al^{3+}	88	-
Ca^{2+}	99	-
Co^{2+}	110	-
Cu^{2+}	0	0
Fe^{2+}	108	-
Fe^{3+}	113	-
Li^{+}	101	-
Mg^{2+}	116	-
Mn^{2+}	89	-
Zn^{2+}	105	-

Enzyme reaction was carried out at 45°C for 1 h in the presence of various metal ions at a final concentration of 1 mM. The metal ions inhibiting enzyme activity were examined again at a concentration of 0.5 mM.

Reaction Products from Inulin

The products obtained from the enzyme reaction in which inulin was added as a substrate were analyzed by HPLC. As shown in Fig. 4, the CFTase produced CF6 and CF7 in an approximate ratio of 4:1 after 6 h incubation, and the yield of cyclofructans was estimated

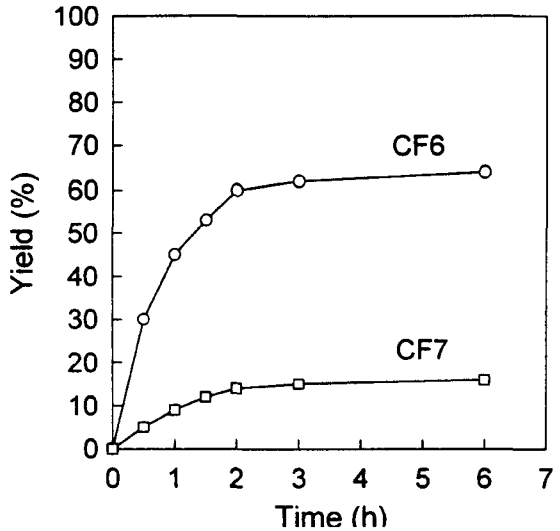

Fig. 4. Formation of cycloinulooligosaccharides from inulin by purified enzyme.
0.1 g dahlia inulin was incubated with 0.5 U of purified enzyme in a total volume of 5 ml at 45°C.

to be up to 80% of the amount of inulin. A small amount of CF8 was also detected in the reaction mixture.

Substrate Specificity

Next, we tested whether the CFTase could produce cyclofructan from such substrates as glucofructo-oligosaccharides and inulins from different sources (Table 3). The enzyme preferred dahlia and jerusalem articoke inulins to chicory inulin. Sucrose (GF), 1-kestose (GF2), and nystose (GF3) were not substrates for the CFTase, but 1-β-fructofuranosyl nystose (GF4) was attacked by the enzyme. The formation of CF6 from GF4 was considered to be due to a disproportionation reaction through an intermolecular transfructosylation.

CFTase Catalyzes Intermolecular Transfructosylation Reactions

We then investigated in detail the intermolecular transfructosylation reactions with the CFTase from *Bacillus macerans* CFC1. Figure 5A shows a typical chromatogram of the cyclization products obtained from the enzymic reaction where inulin was used as a substrate. When 1-β-fructofuranosyl nystose (GF4) was incubated with the CFTase at 37°C for 6 h, CF6 was produced in addition to sucrose (compare Fig. 5C with 5B). This result indicates that CFTase can also catalyze a disproportionation reaction. When sucrose and CF6 were used as a fructosyl acceptor and donor molecule, respectively, oligosaccharides presumed to be glucofructo-oligosaccharides (GF2-GF4) were detected with reduction of sucrose and CF6 after 16 h incubation at 37°C (compare Fig. 5E with 5D). Although GF7, an expected coupling reaction product, was not detected in this assay, reduction in the levels of the reactants (sucrose and CF6) and appearance of new oligosaccharide peaks indicated that a coupling reaction had actually been catalyzed by the enzyme. Moreover, the results described above were not observed in a control reaction where sucrose alone was used as a reactant. This can be explained by the hypothesis that transfructosylation to sucrose occurred at first, after which disproportionation reactions between

Table 3. Substrate specificity of CFTase.

Substrate	Relative activity (%)
Dahlia inulin	100
Chicory inulin	83
Jerusalem articoke inulin	101
Sucrose (GF)	0
1-Kestose (GF_2)	0
Nystose (GF_3)	0
1-β-Fructofuranosyl nystose (GF_4)	16

Reaction mixture contained 2% of substrate and 100 mU of purified enzyme in 50 mM phosphate buffer, pH 7.0. Enzyme reaction was carried out at 45°C for 1 h.

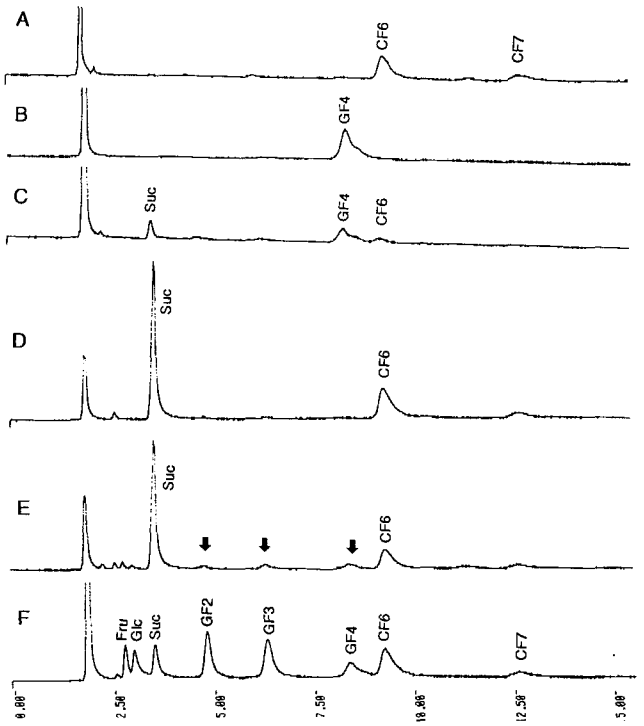

Fig. 5. CFTase from *Bacillus macerans* CFC1 catalyzes disproportionation and coupling reactions besides cyclization reaction.

A. A typical chromatogram pattern of reaction products from inulin as a substrate. B, C. GF4 as both a fructosyl acceptor and a donor was incubated at 37°C for 6 h in the absence (B) or presence (C) of the enzyme. In the presence of CFTase, reduction of GF4 and formation of sucrose and CF6 could be detected. D, E. Sucrose as a fructosyl acceptor was incubated with CF6 as a donor at 37°C for 16 h in the absence (D) or presence (E) of the enzyme. In the presence of CFTase, sucrose was reduced and oligosaccharides (arrows) were produced. F. Mixture of sugars. Glc; glucose, Fru; fructose, Suc; sucrose, GF2; 1-kestose, GF3; nystose, GF4; 1-β-fructofuranosyl nystose, CF6; cycloinulohexaose, CF7; cycloinuloheptaose.

Table 4. Properties of CFTases.

Strain	Molecular mass (kDa)	Optimal temp. (°C)	Optimal pH	Metal inhibitor	Intermolecular trans-fructosylation	Ref.
Bacillus circulans OKUMZ31B	132	40	7.5	Hg^{2+}	Yes	[6]
Bacillus circulans MCI-2554	115	45	7.5	Fe^{2+}, Cu^{2+}	ND	[10]
Bacillus macerans CFC1	110	45	7.5	Ag^{+}, Cu^{2+}	Yes	this work

ND; not determined.

GF7 and sucrose (or GF7) and/or cyclization reaction followed rapidly. Also, in the reaction where 1-kestose and nystose were used as acceptors, the CFTase was observed to catalyze a coupling reaction (data not shown). When intermolecular transfructosylation reactions were carried out at 45°C, the optimal temperature for cyclization reaction, for 3 h, the enzyme was found to catalyze not a coupling reaction, but rather a disproportionation reaction (data not shown), indicating that the optimal temperature for the coupling reaction was different from that for the cyclization reaction. Taken together, the results described above indicate that the CFTase from *Bacillus macerans* CFC1 catalyzes not only a cyclization reaction as a major reaction but also disproportionation and coupling reactions as side reactions.

DISCUSSION

Only two purified CFTases have been reported so far [6, 10]. These two CFTases are produced by two different strains of *Bacillus circulans*. In this work, CFTase from *Bacillus macerans* CFC1 isolated from a soil sample [7] was purified and characterized. Table 4 summarizes properties of CFTases from the *Bacillus* strains mentioned above. The molecular mass (110 kDa) of the enzyme from *Bacillus macerans* CFC1 was similar to that of the CFTase from *Bacillus circulans* MCI-2554. Our enzyme is also functional as a monomer like the other two CFTases. There are, however, differences between our enzyme and the other two CFTases in terms of substrate specificity and effects of metals on enzyme activity. Nystose (GF3) is a substrate for the CFTase from *Bacillus circulans* OKUMZ31B, but the CFTase from *Bacillus macerans* CFC1 can not attack GF3. The activity of CFTase from *Bacillus circulans* MCI-2554 is reported to be greatly inhibited by Fe ions, but our enzyme is slightly activated in the presence of Fe ions.

Cyclodextrin consisting of α-(1→4)-linked cyclic D-glucopyranose has a cone-shaped cavity and can bind neutral molecules via hydrophobic interactions. In contrast, cyclofructan has a characteristic crown ether in the central part of the molecule and can bind cationic molecules via charge-dipole electrostatic interactions [16]. Hence cyclofructan, with a completely different functional structure from cyclodextrin, is considered to have potential in such capacities as a novel host molecule in bioorganic chemistry, an ionophore, and an effective protectant of liposome.

Bacillus macerans CFTase catalyzes not only the cyclization reaction but also coupling and disproportionation reactions in the same manner as CGTase. There have been many studies on transglycosylation by CGTase for improving physicochemical properties of sugars and synthesizing new functional oligosaccharides. They include transglycosylation to glucose [9, 15], stevioside [3], and sugar alcohols [8]. CFTase may also be used for synthesis of a variety of fructosyl sugar derivatives whose physicochemical and functional properties are greatly improved. We will conduct detailed studies on the transfructosylation function of CFTase in the near future.

Acknowledgment

This work was supported by the Korea Science and Engineering Foundation (Project No. 971-0509-049-2). Submission charges were in part by the Center for Advanced Food Science and Technology, Korea University (98024).

REFERENCES

1. Eom, S.-J., Y.-M. Kwon, and Y.-J. Choi. 1995. Molecular cloning of *Pseudomonas* sp. inulinase gene and its expression in *E. coli*. *Kor. J. Appl. Microbiol. Biotechnol.* **23:** 550–555.
2. Haraguchi, K., K. Seki, M. Kishimoto, T. Nagata, T. Kasumi, K. Kainuma, and S. Kobayashi. 1995. Cloning and nucleotide sequence of the inulin fructotransferase (DFA I-producing) gene of *Arthrobacter globiformis* S14-3. *Biosci. Biotech. Biochem.* **59:** 1809–1812.
3. In, M.-J., D.-C. Kim, H.-J. Chae, K.-S. Choi, and M.-H. Kim. 1997. Immobilization of cyclodextrin glucanotransferase and its reaction characteristics regarding transglucosylated stevioside production. *Kor. J. Appl. Microbiol. Biotechnol.* **25:** 305–310.
4. Kawamura, M. and T. Uchiyama. 1989. Formation of a cycloinulo-oligosaccharide from inulin by an extracellular enzyme of *Bacillus circulans* OKUMZ 31B. *Carbohydr. Res.* **192:** 83–90.
5. Kawamura, M. and T. Uchiyama. 1993. Reactions catalyzed by cycloinulo-oligosaccharide fructanotransferase. *Biosci. Biotech. Biochem.* **57:** 343.
6. Kawamura, M. and T. Uchiyama. 1994. Purification and some properties of cycloinulo-oligosaccharide fructanotransferase from *Bacillus circulans* OKUMZ 31B. *Carbohydr. Res.* **260:** 297–304.
7. Kim, H.-Y., J.-B. Park, Y.-M. Kwon, and Y.-J. Choi. 1996. Production of cycloinulooligosaccharide fructanotransferase (CFTase) from *Bacillus* sp. CFC1. *J. Microbiol. Biotechnol.* **6:** 397–401.
8. Kim, T.-K., D.-C. Park, and Y.-H. Lee. 1997. Synthesis of glucosyl-sugar alcohols using glycosyltransferase and structural identification of glucosyl-maltitol. *J. Microbiol. Biotechnol.* **7:** 310–317.
9. Kobayashi, S., H. L. Ashraf, P. Braun, and D. French. 1988. The branched maltooligosaccharides resulting from the action

of *Bacillus macerans* cycloamylose glucanotransferase on 6-O-α-D-glucopyranosyl-cyclomaltohexaose plus D-glucose. *Carbohydr. Res.* **173:** 324–331.
10. Kushibe, S., K. Mitsui, M. Yamagishi, K. Yamada, and Y. Morimoto 1995. Purification and characterization of cycloinulooligosaccharide fructanotransferase (CFTase) from *Bacillus circulans* MCI-2554. *Biosci. Biotech. Biochem.* **59:** 31–34.
11. Kushibe, S., R. Sashida, and Y. Morimoto 1994. Production of cyclofructan from inulin by *Bacillus circulans* MCI-2554. *Biosci. Biotech. Biochem.* **58:** 1136–1138.
12. Laemmli, U. K. 1970. Cleavage of structure protein during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.
13. Park, J. B., Y. M. Kwon, and Y. J. Choi. 1995. Production of inulin fructotransferase (depolymerizing) by *Arthrobacter* sp. A-6. *Kor. J. Appl. Microbiol. Biotechnol.* **23:** 68–74.
14. Park, J. B. and Y. J. Choi. 1996. Purification and characterization of inulin fructotransferase (depolymerizing) from *Arthrobacter* sp. A-6. *J. Microbiol. Biotechnol.* **6:** 402–406.
15. Shiota, M. and S. Kobayashi. 1991. Analyses of α-linked disaccharides of D-glucose by high-performance liquid chromatography. *Carbohydr. Res.* **215:** 203–209.
16. Takai, Y., Y. Okumura, T. Tanaka, M. Sawada, S. Takahashi, M. Shiro, M. Kawamura, and T. Uchiyama. 1994. Binding characteristics of a new host family of cyclic oligosaccharides from inulin: Permethylated cycloinulohexaose and cycloinuloheptaose. *J. Org. Chem.* **59:** 2967–2975.
17. Yokota, A., S. Hirayama, K. Enomoto, Y. Miura, S. Takao, and F. Tomita. 1991. Production of inulin fructotransferase (depolymerizing) by *Arthrobacter* sp. H65-7 and preparation of DFA III from inulin by the enzyme. *J. Ferment. Bioeng.* **72:** 258–261.