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Molecular Characterization of Cycloinulooligosaccharide Fructanotransferase from *Bacillus macerans*

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Cycloinulooligosaccharide fructanotransferase (CFTase) converts inulin into cyclooligosaccharides of β-(2→1)-linked D-fructofuranose by catalyzing an intramolecular transfructosylation reaction. The CFTase gene was cloned and characterized from *Bacillus macerans* CFC1. The CFTase gene encoded a polypeptide of 1,333 amino acids with a calculated M_r of 149,563. Western blot and zymography analyses revealed that the CFTase with a molecular mass of 150 kDa (CFT150) was processed (between Ser389 and Phe390 residue) to form a 107-kDa protein (CFT107) in the *B. macerans* CFC1 cells. The processed CFT107 was similar in its mass to the previously purified CFTase from *B. macerans* CFC1. The CFT107 enzyme was produced by *B. macerans* CFC1 but was not detected from the recombinant *Escherichia coli* cells, indicating that the processing event occurred in a host-specific manner. The two CFTases (CFT150 and CFT107) exhibited the same enzymatic properties, such as influences of pH and temperature on the enzyme activity, the intermolecular transfructosylation ability, and the ability of hydrolysis of cycloinulooligosaccharides produced by the cyclization reaction. However, the thermal stability of CFT107 was slightly higher than that of CFT150. The most striking difference between the two enzymes was observed in their K_m values; the value for CFT150 (1.56 mM) was threefold lower than that for CFT107 (4.76 mM). Thus, the specificity constant (k_{cat}/K_m) of CFT150 was about fourfold higher than that of CFT107. These results indicated that the N-terminal 358-residue region of CFT150 played a role in increasing the enzyme's binding affinity to the inulin substrate.

Inulin is a polyfructan consisting of a linear β-(2→1)-linked polyfructose chain with a terminal glucose residue, thus yielding a sucrose unit at the putative reducing end, and serves as a carbohydrate reservoir in various plants, such as chicory, dahlia, and Jerusalem artichoke. Several types of inulin-decomposing enzymes have been reported, including β-D-fructan fructanohydrolase (EC 3.2.1.7; endoinulinase) (2), β-D-fructofuranosidase (EC 3.2.1.26; exoinulinase) (11), inulin fructotransferase (depolymerizing) (EC 2.4.1.93) (13), and cycloinulooligosaccharide fructanotransferase (4).

Cycloinulooligosaccharide fructanotransferase (CFTase) converts inulin into cyclooligosaccharides consisting of six to eight β-(2→1)-linked D-fructofuranoses (cycloinulohexaose [CF6], cycloinuloheptaose [CF7], and cycloinulooctaose [CF8]). Cyclofructans have a characteristic crown ether in the central part of the molecule and can bind cationic molecules via charge-dipole electrostatic interactions (15). Accordingly, cyclofructans are considered to have potential capacities as a novel host molecule in bioorganic chemistry, an ionophore, and an effective protectant of liposome. To date, three CFTases have been isolated and characterized, one each from *Bacillus circulans* OKUMZ31B (5), *B. circulans* MCI-2554 (10), and *Bacillus macerans* CFC1 (8). Only the CFTase gene (*cft*) from *B. circulans* MCI-2554 has been cloned and sequenced (GenBank/EMBL/DDBJ accession no. D87672) (3). The CFTase of *B. circulans* MCI-2554 was deduced to consist of 1,503 amino acids with a calculated molecular mass of 167 kDa. Interestingly, the molecular mass of the purified CFTase from the culture supernatant of the *Bacillus* strain was reported to be 110 kDa (10). However, there was no report to explain why the calculated molecular mass of the cloned CFTase is different from that of the purified CFTase.

Previously, we isolated a strain of *B. macerans* CFC1 from soil which produced an extracellular CFTase (7), and subsequently, the enzyme was purified and characterized (8). The CFTase from *B. macerans* CFC1 had a molecular mass of 110 kDa and was catalytically active as a monomer. This enzyme was also found to catalyze coupling and disproportionation reactions through an intermolecular transfructosylation. Therefore, this enzyme could be used for synthesis of a variety of fructosyl sugar derivatives whose physicochemical and functional properties are different. Here, we describe the cloning, characterizing, and processing of the CFTase from *B. macerans* CFC1.

Cloning of the *cft* gene from *B. macerans* CFCl. We determined the N-terminal amino acid sequence of the previously purified CFTase of *B. macerans* CFC1 to be FQASDRGT IFYLNL. Based on this N-terminal sequence and the internal peptide sequence (YHLFYQMNPQG) of the CFTase from *B. circulans* MCI-2554 (10), the following primers were synthesized: C1 [5′ GA(T/C) CGI GGI ACI ATI TT(T/C) TA(T/C) CT 3′; I, inosine] and C5 [5′ CAT (T/C)TG (A/G)TA (A/G) AA IA(A/G) (A/G)TG (A/G)TA 3′]. Using these primers (20 pmol of each) and 100 ng of the genomic DNA as a template, a PCR was done for 30 cycles (94°C for 50 s, 46°C for 50 s, and 72°C for 50 s). An amplified 0.9-kb DNA fragment was obtained and confirmed to be a part of the *cft* gene, based on comparison with the *cft* gene of *B. circulans* MCI-2554 (GenBank/EMBL/DDBJ accession no. D87672). To clone the entire *cft* gene of *B. macerans* CFC1, the chromosomal DNA was digested with *Sal*I and *Kpn*I and Southern blotting was performed using the 0.6-kb *Bam*HI fragment of the PCR product as a probe. A genomic DNA fragment with a size of about 5.0

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TABLE 1. Recombinant plasmids used in this work

Plasmid	Description
pCFM	Derivative of pUC119 carrying a 4,896-bp *Sal*I-*Kpn*I fragment containing *cft*
pCFP	Derivative of pCFM deletion from nt 1 to 1037 of the 4,896-bp *Sal*I-*Kpn*I fragment
pCFQ	Derivative of pCFM deletion from nt 408 to 1237 of the 4,896-bp *Sal*I-*Kpn*I fragment
pCFMΔCRE1	Derivative of pCFM deletion from nt 1 to 261 of the 4,896-bp *Sal*I-*Kpn*I fragment
pCFMΔCRE2	Derivative of pCFM deletion from pCFM deletion from nt 1 to 365 or 4,896-bp *Sal*I-*Kpn*I fragment
pCFMHis	Derivative of pCFM carrying the CFT150 gene with six His at the C terminus
pECFTN	Derivative of pET23a carrying the CFT107 gene with six His at the C terminus

kb was detected as hybridizing with the probe DNA. Then, a genomic library was constructed in *Escherichia coli* DH5α cells by using the chromosomal DNA fragments which were obtained from the *Sal*I and *Kpn*I digestion (from 4.3 to 6.5 kb) and the pUC119 vector. White colonies on the Luria-Bertani (LB) agar containing 50 μg of ampicillin/ml plus X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and isopropyl-β-D-thiogalactopyranoside (IPTG) were selected and screened by colony hybridization. The recombinant plasmid isolated from the positive clone was named pCFM (Table 1).

On the other hand, the *E. coli* DH5α/pCFM cells grown in the LB medium at 37°C overnight produced CFTase extracellularly as well as intracellularly, and the ratio of the extracellular to the intracellular activity was about 7:3. The extracellular production of CFTase was confirmed by observing the clear zone formed around the colonies of the recombinant *E. coli* strain on the LB agar supplemented with ampicillin and inulin from dahlia (Fig. 1).

Characterization of the *cft* gene. The complete nucleotide sequence of the 5.0-kb *Sal*I-*Kpn*I insert was determined (Fig. 2). The deduced N-terminal amino acid sequence (FQASDRG TIFYLNL) matched perfectly with the N-terminal sequence of the purified CFTase from *B. macerans* CFC1 (corresponding to nucleotide [nt] positions 1618 to 1659). An ATG codon (at position nt 1495) was found at the position 41 amino acid residues upstream of the N terminus of the purified enzyme. However, neither a potential signal peptide between the ATG codon and the determined N-terminal sequence nor any potential promoter sequence immediately upstream of the ATG codon could be recognized. Instead, a long extended open reading frame from nt 451 to 4452 was identified, which could encode a 150-kDa protein of 1,333 amino acid residues containing a typical signal peptide at the N terminus. This result raises an interesting question of whether the long structural gene is actually expressed in the recombinant *E. coli* pCFM strain to produce the deduced 150-kDa protein with the CFTase activity.

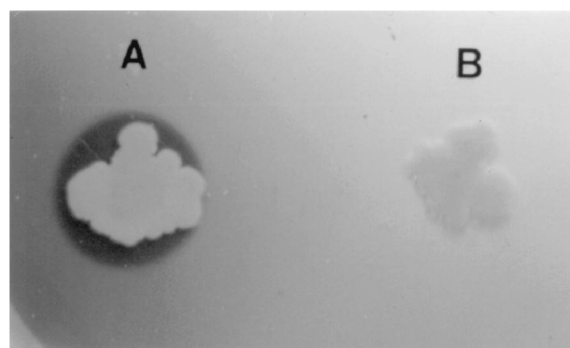

FIG. 1. Clear-zone formation of recombinant *E. coli* DH5α cells expressing *cft* gene. Cells were incubated at 37°C for 2 days in LB agar medium containing 50 μg of ampicillin/ml and partially solubilized inulin. (A) *E. coli* DH5α/pCFM; (B) *E. coli* DH5α/pUC119.

To address this question, the following experiments were performed. Firstly, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the cell extract of *E. coli* pCFM was performed, and an apparent protein band was shown to be at 150 kDa, with no recognizable protein band at 110 kDa (data not shown). Secondly, we constructed two derivatives of pCFM (pCFP, deleted from nt 1 to 1037, and pCFQ, deleted from nt 408 to 1237) in which some upstream regions of the N terminus of the purified CFTase had been deleted. The deleted plasmids were designed to contain a sufficiently large enough portion of the *cft* gene for synthesis of the 110-kDa protein (from Phe390 to Asn1333, named CFT107). Nevertheless, the CFTase activity was not detectable from both the *E. coli* pCFP and *E. coli* pCFQ cells. These results imply that the *cft* gene expressed in the *E. coli* cells encodes only the protein of large size (150 kDa, named CFT150). Thus, to define the precise open reading frame for CFTase, we purified the CFTase produced by the recombinant *E. coli* cells carrying the plasmid pCFMHis, which encodes the CFTase that is His tagged at its C terminus, and determined its N-terminal amino acid sequence. The molecular masses of the purified His-tagged CFTase from both the intracellular and extracellular fractions were estimated to be about 150 kDa (data not shown). The N-terminal sequences of the intracellular and extracellular CFTases were MRKVKRGK and SENRTVAGET, respectively. The recombinant *E. coli* cells carrying the plasmid pCFMΔCRE2 (deleted from position nt 1 to 365) produced a similar level of CFTase activity to that of the plasmid pCFM, indicating that the 86-bp upstream sequence of the translation start site is sufficient for transcription of the *cft* gene in the *E. coli* cells. A putative Shine-Dalgarno sequence (AGGAGG) was located at 8 bp upstream of the translation initiation codon (ATG). The putative −35 (TTCG AA) and −10 (TATAA) elements were identified within the 86-bp upstream sequence. Taken together, the *cft* gene was confirmed to encode a polypeptide of 1,333 amino acids, including a signal peptide of 31 amino acids at the N terminus (Fig. 2). The molecular mass and pI value of the CFTase were 149,563 Da and 4.9, respectively. Notably, the deduced molecular mass of the truncated 944-residue enzyme (Phe390 to Asn1333) was 106,979 Da. This molecular mass agreed well with that of the purified CFTase from *B. macerans* CFC1 (110

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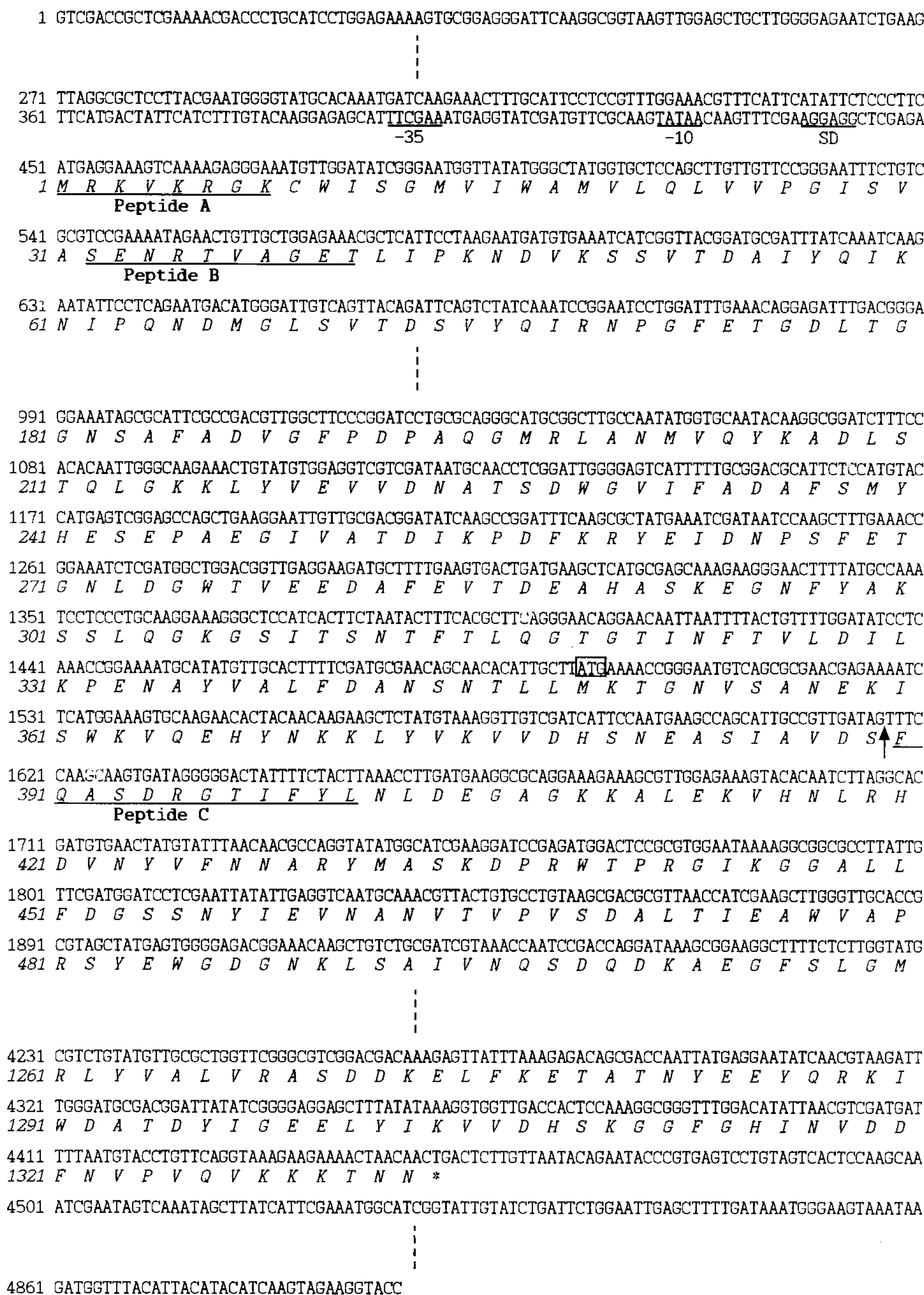

FIG. 2. Nucleotide and deduced amino acid sequences of *B. macerans* CFC1 CFTase. Partial nucleotide and deduced amino acid sequences of CFTase are presented. The complete sequence of the *cft* gene in a 4,896-bp *Sal*I-*Kpn*I fragment was assigned at the GenBank database under accession no. AF222787. The sequences of peptide A (the intracellularly purified CFTase from *E. coli* pCFMHis), peptide B (the extracellularly purified CFTase from *E. coli* pCFMHis), and peptide C (the extracellularly purified CFTase from *B. macerans* CFC1) were determined by N-terminal sequencing. The vertical arrow indicates the proteolytic cleavage site of the protein. An ATG codon at nt 1495 marked by a box.

kDa, as determined by SDS-PAGE). The deduced amino acid sequence of the CFTase showed an 81.5% identity with that of the *B. circulans* MCI-2554 CFTase (data not shown).

CFT107 produced by *E. coli* reveals a CFTase activity. To confirm whether CFT107 (residues 390 to 1333) produced by the recombinant *E. coli* actually has CFTase activity, the plasmid pECFTN was constructed by using a pET23a expression vector and the PCR cloning technique. The *cft* gene on the recombinant plasmid was designed to encode the same protein as the purified CFTase from *B. macerans* CFC1, except that it had an ATG start codon and six His codons at the C terminus. When *E. coli* BL(DE3) carrying pECFTN was induced with 0.5 mM IPTG at 37°C in the LB medium, most of the His-tagged CFT107 protein was detected in the intracellular insoluble fraction. Induction at 20°C for 8 h with 0.5 mM IPTG resulted in an increased level of the intracellular soluble fraction of the

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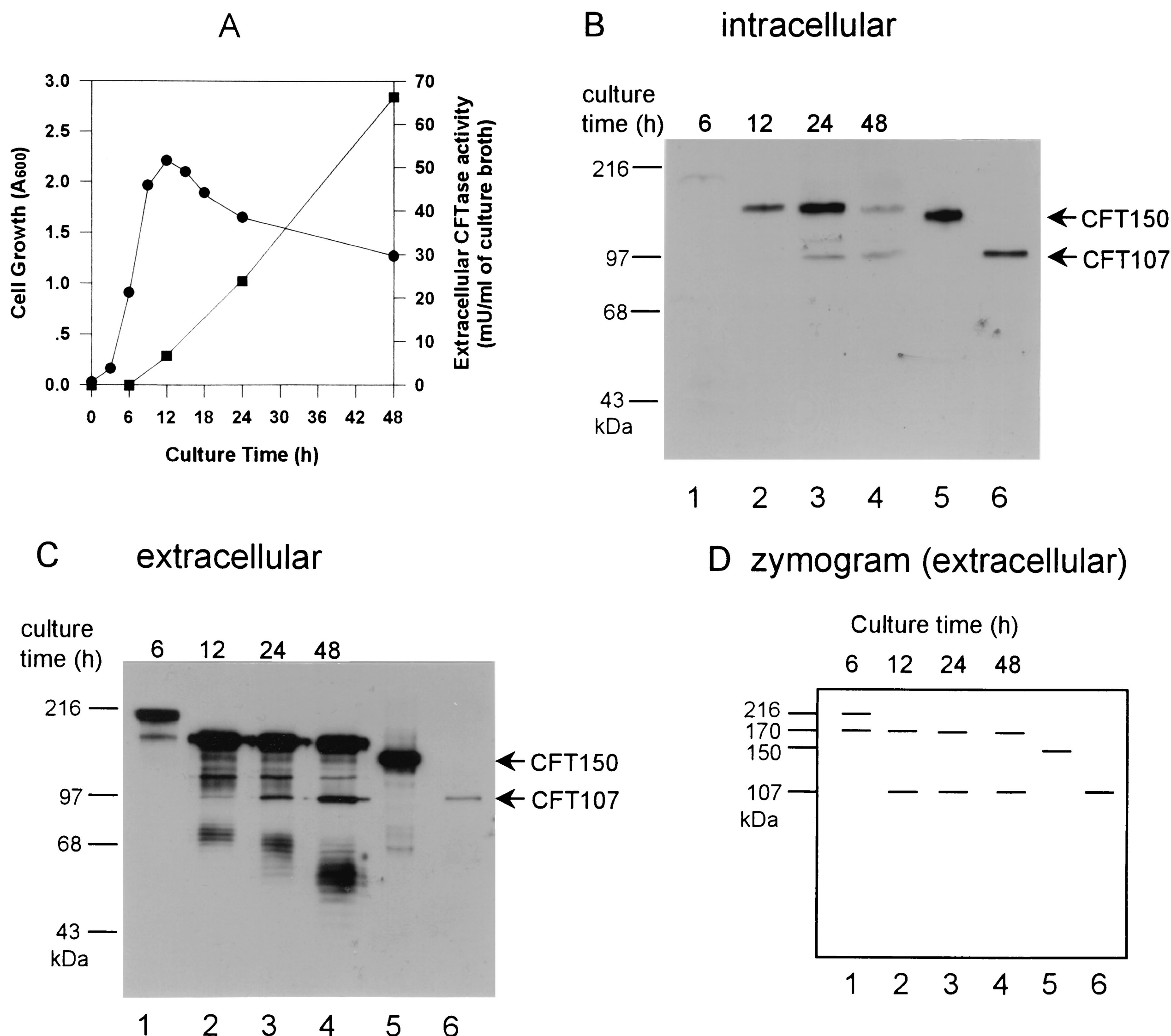

FIG. 3. Expression pattern of the *cft* gene of *B. macerans* CFC1. (A) Cell growth (●) and extracellular CFTase activity (■). Cells were grown in the optimal production medium containing 2% inulin at 37°C. (B and C) Western blot analyses of intracellular (B) and extracellular (C) CFTase. Intracellular samples were prepared as follows. The harvested cells taken at the culture time were adjusted to an equal cell concentration by resuspending with 50 mM phosphate buffer (pH 7.5) and lysing by sonication. Equal amounts of proteins were subjected to SDS–8% PAGE. The extracellular samples were 10-fold concentrated by Microcon-10 (cutoff, 10,000; Amersham). Equal volumes of samples were subjected to SDS–8% PAGE and transferred to nitrocellulose membranes. The transferred proteins were probed with the polyclonal rabbit anti-CFT150 antibody and detected using enhanced chemiluminescence (Amersham). (D) Zymogram of the extracellular fraction. Samples were electrophoresed on an 8% native-polyacrylamide gel containing incompletely solubilized 2% (wt/vol) inulin. The electrophoresed gel was washed two times for 15 min with 50 mM phosphate buffer (pH 7.5). The washed gels were then incubated in the same buffer at 37°C. The activity bands appeared as transparent bands on the gel. The zymogram was redrawn. Lane 1, 6 h sample; lane 2, 12 h; lane 3, 24 h; lane 4, 48 h; lane 5, purified His-tagged CFT150; lane 6, purified His-tagged CFT107.

His-tagged CFT107. The CFTase activity of the crude intracellular soluble fraction was 0.11 U/mg of protein. However, no CFTase activity was detected in the extracellular fraction, indicating that His-tagged CFT107 lacking a signal peptide had failed to excrete into the culture media. His-tagged CFT107 was purified from the crude intracellular soluble fraction by using Ni-nitrilotriacetic acid affinity chromatography (Qiagen), showing a specific activity of 2.16 U/mg.

CFTase is processed in *B. macerans* CFC1 but not in *E. coli*. Previously, the molecular mass of the purified extracellular CFTase of *B. macerans* CFC1 was determined to be 110 kDa. However, the CFTase produced by the recombinant *E. coli* strain had a molecular mass of 150 kDa as described above. Genomic Southern analysis showed that there is only one copy of the *cft* gene in the genome of *B. macerans* CFC1 (data not shown). Hence, to examine whether *B. macerans* CFC1 produces the long transcript of the *cft* gene encoding the 150-kDa protein (CFT150), reverse transcription-PCR was performed using the following primers: the sense-strand primer corresponding to nt 556 to 575 of the cloned 4,896-bp *Sal*I-*Kpn*I

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fragment (RT1, 5′ACTGTTGCTGGAGAAACGCT3′) and the antisense primer corresponding to nt 1637 to 1618 (RT3; 5′CCCCTATCACTTGCTTGGAA3′). A 1,082-bp DNA fragment was yielded from the total RNAs of *B. macerans* CFC1, implying that *B. macerans* CFC1 cells synthesize the transcript encoding CFT150. As described above, CFT107 produced by *E. coli* pECFTN showed a high level of CFTase activity. Together, these results indicate that CFT150 is processed into CFT107 in *B. macerans* CFC1 cells.

Next, to explore the processing of CFTase in *B. macerans* CFC1 in more detail, we analyzed the protein synthesized by the *E. coli* cells carrying the *cft* gene and *B. macerans* CFC1 by using Western blotting and zymography techniques, using both denatured and native gels. Only the CFT150 protein band was visualized from both the intracellular and extracellular fractions of the *E. coli* pCFMHis cells when detected with the probe of monoclonal anti-His antibody (data not shown). The equivalent result was observed with the polyclonal anti-CFT150 antibody that had been used to recognize CFT107 (data not shown). These data demonstrated that the *E. coli* cells carrying the *cft* gene did not synthesize CFT107 but produced only the CFT150. To further understand the processing event of CFTase in the *B. macerans* CFC1 cells, we analyzed the kinetics of CFTase production during the culturing of the *Bacillus* strain. The CFTase activity was detected from the early stationary phase (after 12 h in culture) and increased along with the culture time (Fig. 3A). Western blot analyses showed that CFT150 was also detected from the intracellular fraction after 12 h in culture, and maximum production was observed after 24 h in culture (Fig. 3B). While CFT107 was detected not only from the extracellular samples but also from the intracellular samples, CFT150 was not detected from all the samples of the extracellular fraction (Fig. 3C and D). Instead, two unknown proteins from the extracellular fraction at positions of about 216 kDa (Fig. 3C, lane 1) and 170 kDa (lanes to 4) were observed, and both of the proteins yielded a clear zone on the zymogram (Fig. 3D). Since neither the extracellular CFTase band nor the intracellular CFTase protein band was detected from the 6-h culture, it seems likely that these 216- and 170-kDa proteins may be other types of inulin-degrading enzymes, such as endoinulinase or exoinulinase. The extracellular production of CFT107 was increased along with the culture time, and this is well in accordance with the extracellular CFTase activity profile against the culture time. Taken together, these results demonstrated that CFT150 initially synthesized in *B. macerans* CFC1 was processed into CFT107, possibly by a proteolytic enzyme. Based on the analysis of the determined N-terminal sequence of CFT107 (FQASDRGT IFYLNL) and the deduced CFTase amino acid sequence, the cleavage site was determined to be between Ser389 and Phe390. Since both CFT150 and its presumed N-terminal cleavage product with a mass of about 40 kDa were not detected from all the extracellular samples, it is possible that the processing event might have occurred during translocation of the protein across the cell membrane. It is also notable that the processing occurred in a host-specific manner, since CFT150 was observed to be processed in the *Bacillus* cells but not in the *E. coli* cells. As of this time, the precise mechanism by which CFTase was processed is unclear. However, it is tempting to speculate that CFTase may be processed during translocation across the cell membrane by a specific protease residing in the cytoplasmic membrane of *B. macerans* CFC1.

TABLE 2. Enzymatic properties of CFT150 and CFT107

Characteristic	CFT150	CFT107
Ratio of reaction products (CF6:CF7)	4:1	4:1
K_m (mM)[a]	1.56	4.76
Specific activity (U/mg)	3.58	2.16
k_{cat} (S^{-1})	15.3	12.2
k_{cat}/K_m (mM^{-1} s^{-1})	9.80	2.56
Optimal temp (°C)	45	45
Optimal pH	7.5	7.5
Thermostable[b]	Less	More
Inhibition by Cu^{2+} and Ag^{+}	Yes	Yes
Intermolecular transfructosylation[c]	Yes	Yes
Hydrolysis of cycloinulooligosaccharides[d]	Yes	Yes

[a] Inulin from dahlia (Sigma) was used. The molecular weight of inulin was assumed to be 5,400.

[b] CFT107 was about 10 to 30% more stable than CFT150 at a range of 30 to 45°C. Both enzymes were not stable at temperatures of >50°C.

[c] Disproportionation and coupling reactions were tested as described previously (9).

[d] The reaction mixture consisted of 50 μl of 0.3% CF6 containing small amounts of CF7 and 50 μl (50 mU, pH 8.0) of the purified His-tagged CFTase. The reaction was carried out at 37 or 45°C for 6 h. The reaction products were analyzed by high-performance liquid chromatography.

Biochemical properties of CFT150 and CFT107. As a first step to explore the role(s) of the N-terminal 358-residue region of CFT150, biochemical properties of purified His-tagged CFT150 and CFT107 were compared. As shown in Table 2, all the parameters tested, such as the product ratio of the enzymic reaction (CF6:CF7), the optimal pH and temperature, and effects of metal ions on the enzyme activity, were similar for the two enzymes. Furthermore, both CFT150 and CFT107 were found to catalyze the disproportionation and coupling reactions through intermolecular transfructosylation as well as the cyclization reaction through intramolecular transfructosylation. However, the thermostabilities of the two enzymes were slightly different: CFT107 was about 10 to 30% more stable than CFT150 at the range of 37 to 45°C, suggesting that the presumably more compact structure of CFT107 might be responsible for its higher thermostability. Interestingly, a striking difference between the two enzymes was observed in their K_m values; the value for CFT150 (1.56 mM) was threefold lower than that for CFT107 (4.76 mM). The specificity constant (k_{cat}/K_m) of CFT150 was about fourfold higher than that of CFT107, indicating that CFT150 had a higher affinity for the inulin substrate than CFT107. Also, the specific activity of CFT150 was higher (3.58 U/mg) than that of CFT107 (2.16 U/mg). Together, these results illustrate that the N-terminal 358-residue domain of CFT150 might play a role in the substrate binding of the enzyme.

As mentioned, it was confirmed that *B. macerans* CFC1 CFTase catalyzes not only the cyclization reaction but also the disproportionation and coupling reactions. In this study, we examined whether the two CFTases (CFT150 and CFT107) have the ability to hydrolyze cycloinulooligosaccharides (cyclization products). Interestingly, new inulooligosaccharides (di-, tri-, and tetrasaccharides) were detected with reduction of CF6 after a 6-h incubation at 37°C in both reaction mixtures catalyzed by CFT150 and CFT107. Moreover, even at 45°C, the optimal temperature for CFTase activity, both of the CFTases were found to also hydrolyze CF6, even though the reaction rate was very low. This finding demonstrates a new property of

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the CFTase of *B. macerans* CFC1: an ability to catalyze the hydrolysis of cycloinulooligosaccharides.

The current experimental results suggest that the overall structure of CFTase (1,333 residues) consists of three distinguishable domains: an N-terminal signal peptide (residues 1 to 31), an N-terminal cleavable region (residues 32 to 389) as a substrate-binding domain, and a core catalytic domain (residues 390 to 1333). The CFT150 synthesized in the *B. macerans* CFC1 cells was processed into CFT107, which was secreted into the culture media. This implies that the cleaved N-terminal part of CFT150 may have some other role(s) besides the substrate binding function described above. Many proteases, growth factors, and hormones are known to have pro-sequences, mainly at their N-terminal position. Most pro-sequences play roles in the folding of the proteins as an intramolecular chaperon (1, 12, 14), transport of the proteins (6), provision of signals for targeting proteins to subcellular location (9), and maintenance of proteins in an inactive state under unsuitable conditions (6). We are in the process of elucidating the precise role(s) of the cleaved N-terminal domain of CFTase.

Nucleotide sequence accession number. The nucleotide sequence of the *cft* gene reported in this paper has been deposited in the GenBank database and was assigned accession no. AF222787.

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