Recombinant Thermostable Cycloinulo-oligosaccharide Fructanotransferase Produced by *Saccharomyces cerevisiae*

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A truncated fragment of the cycloinulo-oligosaccharide fructanotransferase (CFTase) gene of *Bacillus circulans* MCI-2554 was fused to the prepro secretion sequence of the α-factor and expressed in *Saccharomyces cerevisiae* under the control of the 5′ upstream region of the isocitrate lyase gene of *Candida tropicalis* (UPR-ICL). Efficiently secreted recombinant CFTase protein (yeast CFTase) was purified. Yeast CFTase consisted of three protein molecules, each of which had CFTase activity (yeast CFTase 1 [116 kDa], yeast CFTase 2 [117 kDa], and yeast CFTase 3 [116 kDa]). Yeast CFTase 2 was the major product of the expression system employed and was shown to be N glycosylated by endoglycosidase H treatment. Yeast CFTase 1 was N glycosylated but had a short truncation at its N terminus, while yeast CFTase 3 did not contain an N-glycosylated carbohydrate chain(s). Yeast CFTase 2 showed an optimum pH, an optimum temperature, and a pH stability similar to those of CFTase purified from *B. circulans* but exhibited a significant increase in thermostability. Production of yeast CFTase by the strain which had two copies of the CFTase gene integrated into its chromosomes reached 391 U per liter of culture at 120 h, which corresponded to 8.40 mg of protein per liter, by shake-flask cultivation.

Cycloinulo-oligosaccharides (cyclofructans [CF]) are the cyclic oligosaccharides which consist of six to eight molecules of β-(2→1)-linked d-fructofuranose (CF6, CF7, and CF8). This bowl-shaped structure of CF resembles that of cyclodextrins (cyclic malto-oligosaccharides), which have been used extensively in the medical, food, and chemical fields (17). An analysis of the crystal structure of CF6 revealed that its central skeleton had the same structure as that of 18-crown-6-crown ether (6, 16). Accordingly, CF6 has the ability to form complexes with metal cations (7, 21, 23). The practical application of CF has stabilizing effects on various materials during freezing and thawing, e.g., dough used for bread, liposomes used in the cosmetic and medical fields, and so on.

CF is synthesized from inulin through an intramolecular transfructosylation reaction. Inulin is a polyfructan consisting of a linear (1→2)-linked polyfructose chain with a terminal glucose residue. The enzyme which catalyzes this transfructosylation reaction is cycloinulo-oligosaccharide fructanotransferase (CFTase). At present, two microorganisms, *B. circulans* OKUMZ 31B (6) and *B. circulans* MCI-2554 prepared by the standard method (19). Sequence analysis showed that there was a long open reading frame which encoded a protein of 1,503 amino acid residues (Fig. 1A). The overall structure of the CFTase gene showed that there were four repeat sequences, three of which were located in the N-terminal region (R1 to R3) and one of which was located near the C terminus (R4). Between these repeat sequences, there was a region whose sequence showed similarity to that of invertase (the core region). For production of recombinant CFTase in *S. cerevisiae* (yeast CFTase), we used a truncated fragment of the CFTase gene (ΔK2 fragment) lacking the region encoding the three N-terminal repeat sequences (residues 57 to 558). When the ΔK2 fragment was expressed in *Escherichia coli*, the recombinant proteins produced had significant enzymatic activities and the CFTase activity in the culture supernatant was even higher than when the entire CFTase gene was expressed (unpublished data).

For the secretion of yeast CFTase into the culture medium, the prepro leader sequence of the α-factor precursor (MFA1) was fused to the ΔK2 fragment of the CFTase gene. The prepro secretion sequence of MFA1 was amplified from plas-
mid pLS01 by PCR with the following primers: 5′-AGGTAGGGCTTGACATGAGATCATCTCTTTACGTCAAT-3′ and 5′-TCTCTGAAGATCCAAAGATACCCCTTC-3′. pLS01 has the 1.7-kbp EcoR I-EcoR I fragment of MFα1 inserted at the EcoR I site of pUC13 (2). The amplified fragment was cut with SalI and XbaI and inserted into pUC19, which has an XhoI site instead of an SmalI site. The CFTase gene was amplified from pECF21 K2 fragment of the CFTase gene was introduced into pLS01 by PCR with the following primers: 5′-CGGAATCTCGTCTAGAGCCGAACCGG-3′ and 5′-CGAGTCGAGACTCGAGATCTA-3′.

As there are five potential N-glycosylation sites (N-X-S/T) in the isocitrate lyase gene of C. tropicalis, we concluded that yeast CFTase 1 was an N-terminal amino acid sequence for each molecule was then determined (Fig. 2B) with a protein sequencer (model 610A; Applied Biosystems). After SDS-PAGE, proteins to be analyzed were transferred to a sheet of ProBlott (Applied Biosystems). The N-terminal amino acid sequences of yeast CFTase 2 and yeast CFTase 3 were identical (Ala-Glu-Pro-Gly-Ala-Asp-Ile-Glu-Ala for yeast CFTase 2 and Ala-Glu-Pro-Gly-Ala-Asp-Ile for yeast CFTase 3) and also corresponded to the sequence immediately after the Kex2 protease cleavage site. On the other hand, yeast CFTase 1 had a distinct N-terminal sequence (Glu-Ala-Glu-Ser-Glu), which started 14 amino acids from the first Ala detected in yeast CFTase 2 and yeast CFTase 3.

As there are five potential N-glycosylation sites (N-X-S/T) in yeast CFTase, we treated each yeast CFTase with endoglycosidase H (endo H; New England BioLabs). After endo H treatment, decreases in the molecular masses of yeast CFTase 1 and yeast CFTase 2 were observed, while the molecular mass of yeast CFTase 3 did not change (Fig. 2A, lanes 2, 4, and 6). Moreover, the molecular mass of endo H-treated yeast CFTase 2 was the same as that of yeast CFTase 3. Considering these results along with the N-terminal amino acid sequence of each yeast CFTase, we concluded that yeast CFTase 1 was an...
artefact of yeast CFTase 2 due to limited proteolysis at the N terminus and that yeast CFTase 3 had the same protein moiety as yeast CFTase 2 but did not have an N-glycosylated carbohydrate chain(s).

**Properties of yeast CFTase.** By using purified yeast CFTase 2 protein, enzymatic properties with respect to pH and temperature were analyzed. CFTase activity was assayed as described previously (10). One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of cycloalpinolhexose (CF6) per min. Figure 3A shows the effects of pH on the activity and stability of the enzyme. The optimal pH of yeast CFTase 2 was 8.0, and over 80% of the activity was still detected after incubation for 30 min at pH 6.0 to 10.0, as happened with CFTase of *B. circulans* (8). CFTase activity significantly increased between 24 and 48 h of cultivation and continued to increase gradually even up to 120 h of cultivation. The level of CFTase activity at this point periodically over 120 h. YPE medium was previously shown to be effective in the production of CFTase (12).

**Production of yeast CFTase with a chromosomally integrated expression system.** For the stable production of yeast CFTase, we constructed an *S. cerevisiae* strain in which multiple copies of the CFTase gene were integrated into its chromosomes. A *Pvu*II-*Pvu*II fragment from pWIF3 containing *UPR-ICL*, the α-factor prepro sequence, the ΔK2 fragment of the CFTase gene, and the 3′ noncoding region of the *ICL* gene of *C. tropicalis* was excised and introduced into the yeast integrating vectors pRS403 and pRS404 (20) that had been digested by *Pvu*II to construct pWIF33 and pWIF34, respectively (Fig. 4A). pWIF33 and pWIF34 were both cut by *Bsr*XI and introduced into *S. cerevisiae* W303-1A to construct the CF/HW2A strain, in which two copies of the CFTase gene were transformed. Integration of the CFTase gene was confirmed by Southern blot analysis (9) with an *HIS3* or *TRP1* DNA fragment as a probe (Fig. 4B).

CF/HW2A was cultivated in a flask with YPE medium (1% yeast extract, 2% peptone, 2% [vol/vol] ethanol), and the CFTase activity in the culture supernatant was assayed periodically over 120 h. YPE medium was previously shown to strongly induce *UPR-ICL*-mediated gene expression (25). CFTase activity significantly increased between 24 and 48 h of cultivation and continued to increase gradually even up to 120 h of cultivation. The level of CFTase activity at this point was 391 U per liter of culture, which corresponded to 8.40 mg of CFTase protein per liter of culture (Fig. 5). The mitotic
stability of the integrated exogenous DNA sequence after 120 h of cultivation was estimated by comparing the number of colonies grown on a nonselective plate (YPD) with that grown on a selective plate (SD). With CF/HW2A, the number of His \(^+\) Trp \(^+\) colonies on the SD plate was about 94% of that on the YPD plate. When cultivation in YPE medium was repeated five times, the number of His \(^+\) Trp \(^+\) CF/HW2A colonies on the SD plate was still over 80% of that on the YPD plate.

Our yeast CFTase production system has several advantages for further application of this enzyme. First, as *S. cerevisiae*, which has the GRAS status of microorganisms, does not contain any endotoxins or lytic viruses, it is a suitable host for the production of foreign proteins to be used in pharmaceutical or food production (15, 18). Second, compared with native *B. circulans* CFTase or recombinant CFTase produced by prokaryote expression systems, yeast CFTase can be produced as a glycoprotein, making yeast CFTase a highly thermostable enzyme. This high thermostability is a beneficial property for enzymes used in industry (14). Third, our safe culture medium containing high levels of yeast CFTase can directly be used for the production of CF without purification of the enzyme, because yeast CFTase, like *B. circulans* CFTase, was efficiently secreted into the culture medium. Moreover, production of CFTase can readily be increased by optimizing fermentation conditions; when *S. cerevisiae* is cultivated under optimized conditions, cell densities of 50 to 100 mg of dry cells per ml of culture can be obtained (1). Fourth, *S. cerevisiae* secretes invertase into the culture medium, which helps to hydrolyze sucrose generated from inulin during the enzyme reaction. This process prevents an increase in the concentration of sucrose in the reaction medium, which would otherwise result in high viscosity of the solution and create problems in the purification of CF.

**Nucleotide sequence accession number.** The nucleotide sequence of the CFTase gene from *B. circulans* MCI-2554 was submitted to the GenBank, EMBL, and DDBJ data banks under accession no. D87672.

**FIG. 4.** (A) Structures of pWIF33 and pWIF34. Abbreviations of genes are the same as those used in Fig. 1B. (B) Southern blot analysis of genomic DNAs of strains W303-1A and CF/HW2A. In lanes 1 and 2, HIS3 was used as a probe; in lanes 3 and 4, TRP1 was used as a probe. Lanes 1 and 3, W303-1A genomic DNA digested by *Bst* XI; lanes 2 and 4, CF/HW2A genomic DNA digested by *Bst* XI. The arrow indicates integrated exogenous DNA (9.39 kbp for the HIS3 locus and 9.21 kbp for the TRP1 locus) containing the ΔK2 fragment of the CFTase gene as well as HIS3 or TRP1.

**FIG. 5.** Increases in growth of CF/HW2A and in CFTase activities in the culture supernatant. ○, cell growth; ●, CFTase activity in the culture supernatant.
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REFERENCES


