Cycloinulo-oligosaccharides (cyclofructans [CF]) are the cyclic oligosaccharides which consist of six to eight molecules of β-(2→1)-linked d-fructofranose (CF6, CF7, and CF8). This bowl-shaped structure of CF resembles that of cyclodextrins and its nucleotide sequence was determined. In future uses of CF has been widely examined, and it has been shown that cycloinulo-oligosaccharides (cyclofructans [CF]) are the cyclic oligosaccharides which consist of six to eight molecules of β-(2→1)-linked d-fructofranose (CF6, CF7, and CF8). This bowl-shaped structure of CF resembles that of cyclodextrins. Accordingly, CF6 has the ability to form complexes with metal cations (7, 21, 23). The practical application of CF has been widely examined, and it has been shown that 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 polyfructosan consisting of a linear β-(2→1)-linked polyfructose chain with a terminal glucose residue. The enzyme which catalyzes this transfructosylation is cycloinulo-oligosaccharide fructotransferase (CFTase). At present, two microorganisms, \textit{Bacillus circulans} MCI-2554 and \textit{B. circulans} MCI-2554 prepared by the standard method (19), have been found to produce CFTase.

The CFTase gene from \textit{B. circulans} MCI-2554 was isolated, and its nucleotide sequence was determined. In future uses of CF with drugs or foods, baker’s yeast (\textit{Saccharomyces cerevisiae}) is a good choice for an applicator. Because it is a eukaryote that has GRAS (generally recognized as safe) status, it is suitable for the safe production of foreign proteins (15, 18). We have developed a novel and powerful heterologous gene expression system in \textit{S. cerevisiae} using the 5’ upstream region of the isocitrate lyase gene (UPR-ICL) from an alkanotrophic yeast, \textit{Candida tropicalis} (5, 24, 25).

Here, we report the expression of a truncated fragment of the CFTase gene of \textit{B. circulans} MCI-2554 in \textit{S. cerevisiae} under the control of UPR-ICL and its purification, characterization, and stable production by \textit{S. cerevisiae}.

\textbf{Construction of a plasmid for expression of a truncated form of the CFTase gene.} A DNA fragment containing the CFTase gene was isolated from the genomic library of \textit{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 \textit{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 \textit{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'-'-AAGGAT CCCTGACATGAGATCCCTCAATT-3' and 5'-'-TCTCT AGAATCCAAAAGATACCCCTTC-3'. pLS01 has the 1.7-kbp EcoRI-EcoRI fragment of MgF1 inserted at the EcoRI 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 Smal site. The CFTase gene was amplified from pECF21Δ K21, which contains the ΔK2 fragment of the CFTase gene, with the following primers: 5'-'-CGGAATCTCTGACTGAGGC CGAACCCG-3' and 5'-'-CGAGTGACCTGAGATCTA AGCCTT-3'. The amplified fragment was cut with XbaI and Xhol and placed downstream of the α-factor prepro secretion sequence. The XbaI site located between the α-factor prosequence and the CFTase gene corresponds to the last two amino acids of the α-factor prosequence. Introduction of the XbaI site changed its corresponding amino acid sequence, Lys-Arg, to Ser-Arg, and Lys-Arg is the recognition sequence of XbaI site changed its corresponding amino acid sequence, Lys-Arg, to Ser-Arg, and Lys-Arg is the recognition sequence of XbaI site. The CFTase gene sequence and the CFTase gene correspond to the last two amino acids of the XbaI site. The amplified fragment was cut with XbaI and inserted into pWIF31 (5) to make pWIF31 (Fig. 1B). The expression plasmid, the D XhoI fragment containing the a based technique (4). After site-directed mutagenesis, the Kex2 protease for cleavage of the Arg, to Ser-Arg, and Lys-Arg is the recognition sequence of XbaI site changed its corresponding amino acid sequence, Lys-Arg, to Ser-Arg, and Lys-Arg is the recognition sequence of XbaI site.

**TABLE 1. Purification of yeast CFTase from the culture supernatant**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Sp act (U/mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultrafiltration (YM-10 filter)</td>
<td>10.6</td>
<td>229</td>
<td>21.4</td>
<td>100</td>
<td>1.00</td>
</tr>
<tr>
<td>DEAE-Sepharose CL-6B column chromatography</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yeast CFTase 1 (116 kDa)</td>
<td>0.196</td>
<td>7.33</td>
<td>37.4</td>
<td>3.20</td>
<td>1.75</td>
</tr>
<tr>
<td>Yeast CFTase 2 (117 kDa)</td>
<td>0.454</td>
<td>21.1</td>
<td>46.5</td>
<td>9.21</td>
<td>2.17</td>
</tr>
<tr>
<td>Yeast CFTase 3 (116 kDa)</td>
<td>0.122</td>
<td>6.60</td>
<td>54.1</td>
<td>2.88</td>
<td>2.53</td>
</tr>
</tbody>
</table>

**FIG. 1.** (A) Structure of the CFTase gene. a.a., amino acids; R1 to R4, regions of repeat sequences; CR, a region which has a sequence similar to that of the invertase gene. The thick bar indicates the region included in the ΔK2 fragment. (B) Structure of pWIF31. TERM-ICL, the terminator region of the isocitrate lyase gene of C. tropicalis; CFTase gene (ΔK2), the ΔK2 fragment of the CFTase gene diagrammed in panel A.
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 cycloheximide (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* (10). Figure 3B shows the effects of temperature on the activity and stability of the enzyme. The maximal activity was observed at 45°C, as with *B. circulans* CFTase. Interestingly, yeast CFTase 2 showed much higher thermostability than *B. circulans* CFTase. A high level of activity (62.1% of the initial activity) remained after 30 min of incubation at 70°C, while *B. circulans* CFTase was reported to lose its activity under these conditions (8, 10). Even after incubation at 80 or 90°C, yeast CFTase 2 retained 53.6 or 20.6% of the initial activity, respectively. This high level of thermostability, however, was not observed for yeast CFTase 3; only 10.0% of the initial activity was observed after 30 min of incubation at 70°C. These results indicate that the N-glycosylated carbohydrate chain(s) of yeast CFTase 2 plays an important role in the thermostability of the enzyme. There have been several reports indicating that N-glycosylated enzymes produced in *S. cerevisiae* have increased thermostability compared to that of their unglycosylated counterparts and that removal of the N-linked carbohydrate chain(s) results in a decrease in their thermostability (13, 22).

**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-*Pst*I fragment from pWIF31 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 integrative vectors pRS403 and pRS404 (20) that had been digested by *Pvu*II to construct pWF33 and pWF34, respectively (Fig. 4A). pWF33 and pWF34 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 highly 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).
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.
We thank F. Hishinuma at Mitsubishi Kasei Institute of Life Sciences, Tokyo, Japan, for providing plasmid pLS01.

REFERENCES


