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Improved Bread-Baking Process Using *Saccharomyces cerevisiae* Displayed with Engineered Cyclodextrin Glucanotransferase

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A bread-baking process was developed using a potential novel enzyme, cyclodextrin glucanotransferase[3−18] (CGTase[3−18]), that had previously been engineered to have enhanced hydrolyzing activity with little cyclodextrin (CD) formation activity toward starch. CGTase[3−18] was primarily manipulated to be displayed on the cell surface of *Saccharomyces cerevisiae*. *S. cerevisiae* carrying pδCGT integrated into the chromosome exhibited starch-hydrolyzing activity at the same optimal pH and temperature as the free enzyme. Volumes of the bread loaves and rice cakes prepared using *S. cerevisiae*/pδCGT increased by 20% and 45%, respectively, with no detectable CD. Retrogradation rates of the bread and rice cakes decreased significantly during storage. In comparison to the wild type, *S. cerevisiae*/pδCGT showed improved viability during four freeze−thaw cycles. The results indicated that CGTase[3−18] displayed on the surface of yeast hydrolyzed starch to glucose and maltose that can be used more efficiently for yeast fermentation. Therefore, display of an antistaling enzyme on the cell surface of yeast has potential for enhancing the baking process.

KEYWORDS: Antistaling enzyme; cyclodextrin glucanotransferase (CGTase); retrogradation; yeast; cell-surface display; δ-integration

INTRODUCTION

For many years, genetically engineered baker's yeast has been studied for breeding, fermentation capability, and stress tolerance (*1*−*4*). In particular, cell surface display has been applied in research including peptide libraries and antibody targeting on the surface of bacteriophages and bacteria, which can be used for the screening of antigens and immunology (*5*, *6*). Many investigators have explored the system for cell surface display in application to foods, alcoholic beverages, and medicines. In practical use, the most suitable microorganism is *Saccharomyces cerevisiae*, which is "generally regarded as safe (GRAS)" because it has been used for brewing and baking for a long time.

After bread is leavened, a series of physicochemical changes develops in the loaf that leads to deterioration of quality during storage. A change in crumb firmness, called retrogradation, is one of the major factors affecting consumer acceptance. Similarly, *Jeungpyun*, a kind of traditional Korean rice cake, in which rice powder is fermented with yeast, has not been widely developed to industrial scales because of its high starch retrogradation rate. Therefore, several enzymes such as α-amylase and cyclodextrin glucanotransferase (CGTase) have been included in efforts to preserve the softness of bread after longer storage periods (*7*, *8*).

CGTases (EC 2.4.1.19) shows the highest structural homology with Novamyl, a commercial antistaling enzyme. Unlike ordinary α-amylase from glycoside hydrolase family 13, Novamyl and CGTase have two additional domains (D and E), and especially, the E-domain was reported as the starch-binding domain. Therefore, they are advantageous in binding to granular starch (*9*). However, CGTases produce various cyclodextrins (CDs) via intramolecular transglycosylation of maltooligosaccharides. CDs produced by the action of CGTase in bread could be a problem in countries where CDs are not allowed as food additives. Because of this restriction, the development of mutant CGTases that produce large amounts of maltooligosaccharides but less CD has been attempted. Recently, we reported improvement of CGTase from alkalophilic *Bacillus* I-5 as an antistaling agent for bread by modifying the hydrolyzing and cyclization activity of the enzyme by error-prone PCR (*10*).

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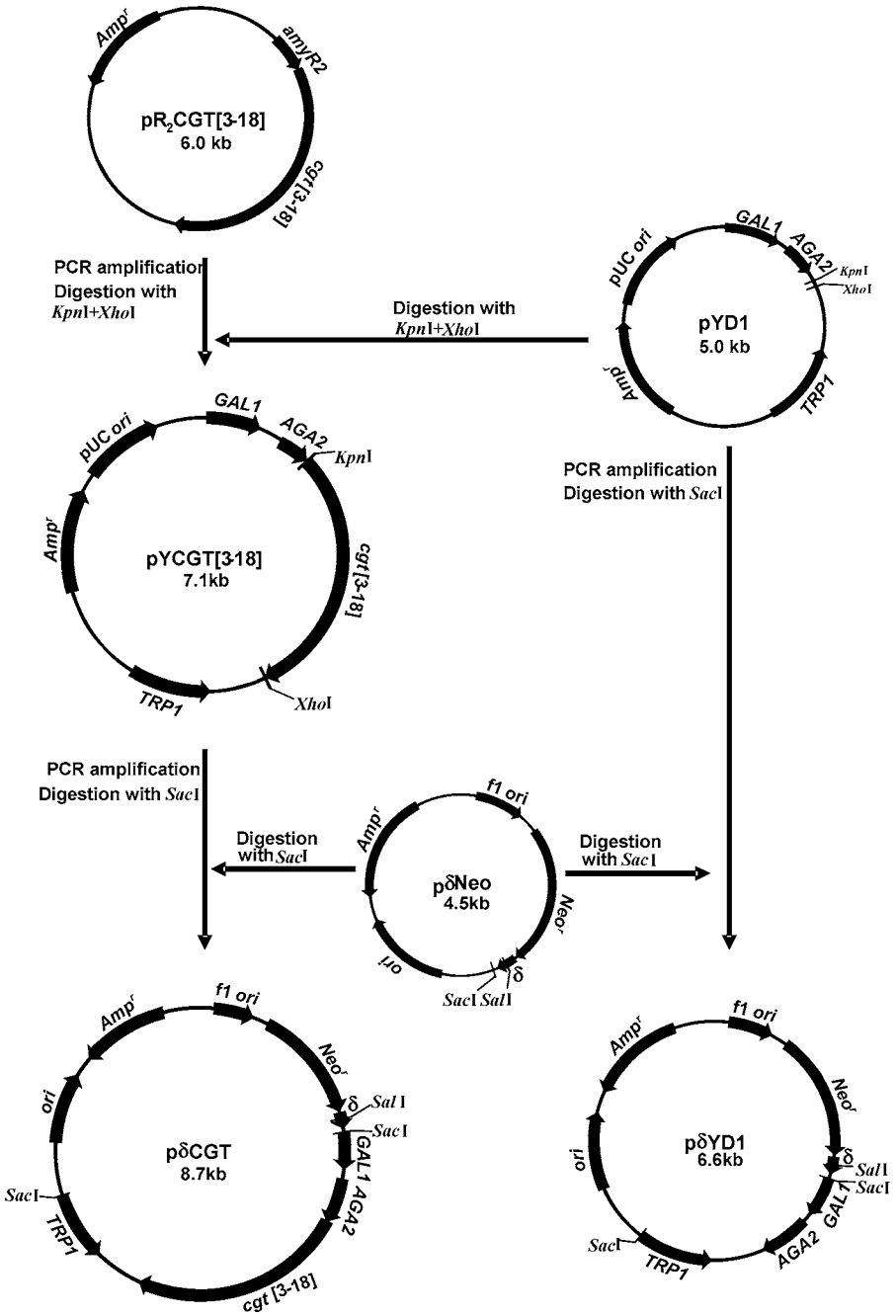

Figure 1. Construction of rDNAs for cell display. The 2.1 kb DNA fragment of the *cgt*[3–18] gene truncated in its signal sequence was amplified using synthetic primers containing a *Kpn*I and an *Xho*I site. The resulting PCR product was digested with the enzymes and ligated into the corresponding sites on pYD1. The 4.2 kb and 2.1 kb DNA fragments containing the region between *GAL1* and *TRP1* were amplified using the synthetic primers containing a *Sac*I site from the templates pYCGT and pYD1, respectively, by PCR. The PCR products were digested with *Sac*I and ligated to p*δ*Neo at the corresponding site.

In this study, CGTase[3−18] was used as a target protein displayed on the cell surface to develop a baker's yeast with antistaling characteristics. The enzymatic properties of the engineered yeast were analyzed, and the engineered *S. cerevisiae* was evaluated as a baker's yeast having antistaling effects on bread and rice cakes.

MATERIALS AND METHODS

Microorganisms, Plasmids, and Culture Conditions. *Escherichia coli* DH5 [F^-, *lacZ*Δ*M15*, *hsdR17*(r^-m^-), *gyrA36*] was used as a host for gene cloning. *S. cerevisiae* EBY100 [*trp1*, *leu2*Δ*1*, *his3*Δ*200*, *pep4*:: *HIS2*, *prb*Δ*1.6R an1*, *GAL*] was used in the expression and display of CGTase[3−18]. The *cgt*[3−18] gene was isolated from pR$_2$CGT[3−18] (*10*). pYD1 (Invitrogen, Carlsbad, CA) was used to express the *cgt*[3−18] gene in *S. cerevisiae*. An integration vector, p*δ*Neo (*11*), was kindly provided by Professor Jin-Ho Seo (Seoul National University).

E. coli was cultured in LB medium (1% bacto−tryptone, 0.5% yeast extract, 0.5% NaCl) at 37 °C, and *E. coli* transformants were grown in LB medium supplemented with ampicillin (100 μg/mL). Yeast transformants were selected and maintained on a YNB-GS plate [0.67% yeast nitrogen base without amino acid (YNB; Sigma Aldrich, St. Louis, MO), 0.19% yeast synthetic drop-out medium supplement without tryptophan (YNB; Sigma Aldrich, St. Louis, MO), 2% galactose, 1% starch, and 1.5% agar]. The seed culture of *S. cerevisiae* grown in YNB

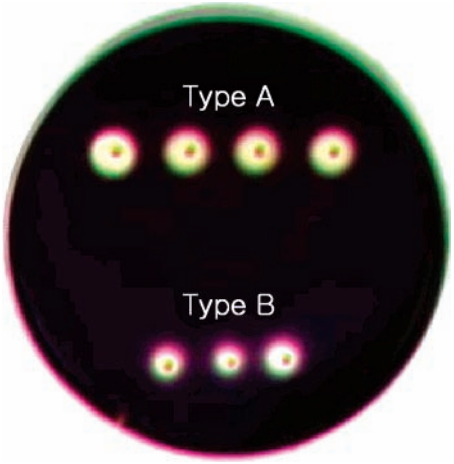

Figure 2. Agar plate assay of the amylolytic activities of *S. cerevisiae*/pδCGT (**A**) and *S. cerevisiae*/pYCGT (**B**).

medium was transferred to a YPG medium (1% yeast extract, 2% peptone, and 2% galactose) and incubated at 25 °C for 48 h with shaking (200 rpm).

Construction of pYCGT[3−18] and pδCGT[3−18]. The *cgt*[3−18] gene on pR_2CGT[3−18] was amplified by polymerase chain reaction (PCR) using the primers KpnI-N (5′-CACCATCACCAGGTACCTATGAGGAAAGAAGCC-3′) and XhoI-C (5′-GTAACAAGCGGC CTCGAGCCTCTACCCGGAGCC-3′). A 2.1 kb *Kpn*I-*Xho*I fragment of the PCR product was subcloned into pYD1, a yeast display vector, and designated pYCGT (**Figure 1**). Another set of primers including Int_F (5′-ACCCTCACTAAAGAGCTCAAAAGCTGGCTAG T-3′) and Int_R (5′-GTGATTAAGCACACAGAGCTCGCTTGGAGTATG-3′) was then used for the amplification of the region between *GAL1* and *TRP1* on pYCGT and pYD1. The PCR mixture was composed of 0.25 mM of each dNTP, 5 pmol of each primer, and 5 U of ExTaq polymerase (Takara, Shuzo, Japan). PCR was performed using an automatic thermal cycler (GeneAmp 9600; Perkin-Elmer, Norwalk, CT) for 30 cycles; each cycle consisted of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 2 min, and a final extension at 72 °C for 7 min. Two (4.2 kb and 2.1 kb) DNA fragments amplified from pYCGT and pYD1, respectively, by PCR were digested with *Sac*I and then ligated to pδNeo at the corresponding sites. The resulting recombinant DNAs (rDNA) designated as pδCGT and pδYD1 (**Figure 1**) were transformed into *E. coli* DH5α (*12*) and subsequently to yeast using an alkali-cation kit (Bio101, Palo Alto, CA) according to the manufacturer's instructions.

Enzyme Assay. The hydrolytic activity of CGTase was measured by the DNS method (*13*). CGTase[3−18] or CGTase[3−18]-displaying cells were incubated with 0.5% (w/v) soluble starch solution in 50 mM sodium acetate buffer (pH 6.0) at 50 °C, and the reaction was carried out at 50 °C for 10 min. The reaction samples were boiled for 10 min to end the reaction. After centrifugation (10000*g*, 5 min), 0.5 mL of supernatant was added to 0.5 mL of the DNS solution (10.6 g of 3,5-dinitrosalicylic acid, 19.8 g of NaOH, 306 g of sodium potassium tartrate, 1416 mL of distilled water, 7.6 mL of phenol, and 8.3 g of sodium metabisulfite). The solution was boiled for 5 min and cooled immediately under running tap water; absorbance of the solution was measured at 575 nm (Ultraspec III; Pharmacia, Uppsala, Sweden). The blank was run in the same way but without the enzyme. One unit (U) of hydrolyzing activity was defined as the amount of enzyme that split a 1 μmol equiv of glycosidic bond in soluble starch in 1 min.

Freeze−Thaw Stability. Yeast viability during the freeze−thaw process was examined. Dry cell mass concentration was measured using a spectrophotometer at 600 nm. Equal amounts of wild-type and *S. cerevisiae*/pδCGT culture were stored at −20 °C overnight, and then thawed for 60 min at 27 °C in an incubator. Thawed samples diluted to 10^{-7} were plated on YPD agar (2% peptone, 1% yeast extract, 2% glucose, and 1% agar) and incubated at 30 °C for 60 h. Relative viability was calculated as the ratio of number of live cells to total number of cells at a given point in time.

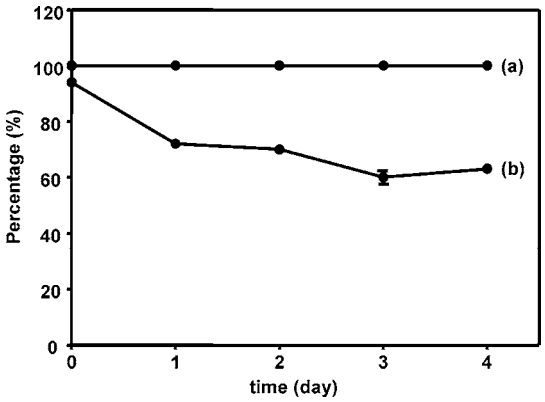

Figure 3. Stability of pδCGT (**a**) and pYCGT (**b**) in the recombinant yeasts.

Bread and Rice Cake Preparation. Bread loaves were baked using White Pan Bread Mix II (CJ Co., Seoul, Korea) as described previously (*8*). Instead of commercial yeast, 2.5 g of recombinant *S. cerevisiae*/pδYD1 or *S. cerevisiae*/pδCGT was used for baking. Bread loaves were stored at 4 °C in polyethylene bags. In rice cake preparation, all ingredients (rice flour, 100 g; sugar, 15 g; water, 60 g; salt, 1 g; *S. cerevisiae*/pδCGT, 1.5 g) were mixed and fermented at 35 °C for 4 h. The fermented rice flour (15 g) was placed in plastic cups (25 mm diameter and 30 mm deep) and steamed for 30 min in an electronic cooker followed by cooling for 30 min. Rice flour fermented with *S. cerevisiae*/pδYD1 was cooked and prepared as a control. The volume of each bread loaf and rice cake was measured by the rice grain displacement procedure.

Analysis of CD in Bread Loaves. Bread crumbs (10 g) were extracted in 100 mL of distilled water by stirring vigorously for 1 h at room temperature. The mixture was centrifuged at 7000*g* for 10 min; the supernatant was diluted with distilled water (1:99, v/v) and filtered through a 0.45 μm filter (Millipore, Bedford, MA). The supernatant (25 mL) was dried using a freeze-dryer (Ilshin Engineering Co., Seoul, Korea) and then dissolved in 5 mL of water. The concentration of CD in the bread was analyzed using high-performance liquid chromatography (HPLC). The HPLC system used in this study (SLC-100, Samsung Electronics Inc., Su-Won, Korea) was equipped with a Linchrosorb NH_2 column (10 μm, 4 mm × 250 mm; Merck, Darmstadt, Germany) and a differential refractometer (Waters 400R; Waters, Milford, MA). The samples were eluted at a flow rate of 1.0 mL/min using a mixture of acetonitrile and water (65:35, v/v).

Texture Profile Analysis (TPA). TPA of bread (1.5 cm × 1.5 cm × 1.5 cm) and rice cake (15 g) was performed using a texture analyzer (TA-XT2i, Stable Micro System, Surrey, U.K.) at room temperature. A two-cycle compression, force-versus-time program was used at a test speed of 2 mm/s and a rate of 50% strain using a cylinder plunger with a diameter of 50 mm. Parameters of the TPA curve were determined as described by Bourne (*14*) and defined by Munoz (*15*).

Differential Scanning Calorimetry (DSC). The retrogradation rate of each loaf was determined using DSC 120 (Seiko Co., Chiba, Japan) at days 3 and 7 during the storage of bread at 4 °C (*12*). The DSC was calibrated with indium (156.6 °C, 28.591 J/g) and tin (232.2 °C, 60.62 J/g). Distilled water was used as a reference. Samples (10 mg) were weighed and hermetically sealed in aluminum pans. The pans were then heated from 20 to 130 °C at a rate of 5 °C/min. The degree of retrogradation was expressed as the enthalpy calculated from the area of the endothermic peak between 40 and 80 °C.

RESULTS AND DISCUSSION

Expression of pYCGT and pδCGT in Yeast. The mutant CGTase gene, *cgt*[3−18], was amplified by PCR with its signal sequence truncated and ligated to pYD1 such that the gene was fused with the *AGA2* gene regulated by the *GAL1* promoter to allow secretion and display of CGTase[3−18]. The resulting plasmid, pYCGT, was transformed into *S. cerevisiae*, an auxotroph for galactose and tryptophan, and the transformants

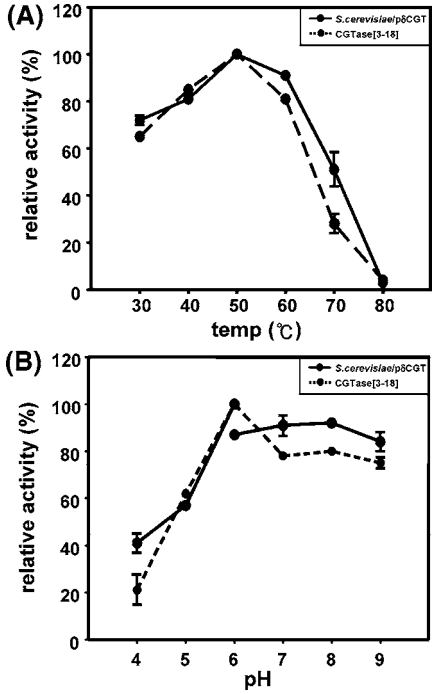

Figure 4. Temperature (**A**) and pH (**B**) profiles of *S. cerevisiae*/pδCGT.

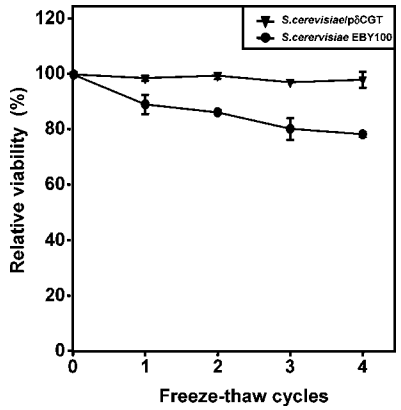

Figure 5. Relative cell viability of the recombinant yeasts during the freeze–thaw process.

were selected on a YNB plate for plasmid persistence. Transformants harboring pYD1 or pYCGT, and the host cells inoculated on YPGS agar plates containing starch, were incubated at 30 °C for 2 days, after which iodine solution was poured over the plates. The results showed that only the cells harboring pYCGT formed a halo around the colonies, whereas no halo formation was observed around the host cells and the cells harboring the vector, pYD1. CGTase[3−18] was successfully displayed and active on the surface of *S. cerevisiae*. However, the size of the clear zone varied among transformants, and one of them lost its activity during nonselective culture. This indicated that the rDNA in an episomal state was not stable, and consequently, levels of gene expression varied. The results implied that the strategy of enzyme display was not appropriate for the purpose of this study. The recombinant yeast cells resulted in slight improvement in the antistaling effect during storage of the bread loaves, but the improvement was insignificant and irreproducible (data not shown).

To keep the foreign DNA stable and enhance the expression of CGTase[3−18] in yeast for industrial application, the δ-integration method was introduced (*16*, *17*). The δ-sequences are the long terminal repeats of *S. cerevisiae* retrotransposon

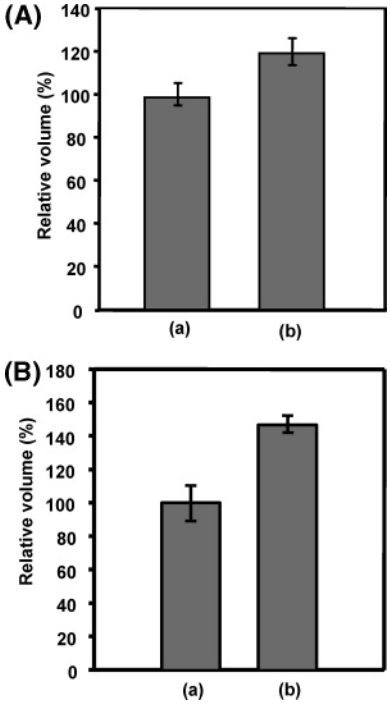

Figure 6. Relative volumes of bread loaves (**A**) and rice cakes (**B**) prepared using *S. cerevisiae*/pδYD1 (**a**, control) and *S. cerevisiae*/pδCGT (**b**).

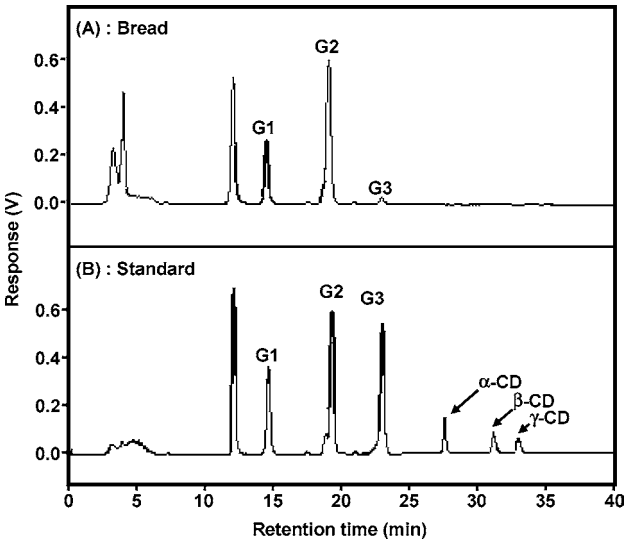

Figure 7. HPLC analysis of maltooligosaccharides in bread loaves baked using *S. cerevisiae*/pδCGT (**A**). The standard molecules were glucose (G1), maltose (G2), maltotriose (G3), and α-, β-, and γ-CDs (**B**).

Ty (*18*, *19*). About 425 copies of δ-sequences that are either Ty associated or at sole sites exist in the haploid yeast genome (*18*). For this reason it is possible to make many copies of heterologous genes integrated into the yeast chromosomes using plasmids carrying the sequence (*20*, *21*). Thus, pδNeo was employed in this study to introduce the *cgt*[3−18] gene into the yeast chromosome (**Figure 1**). The recombinant plasmids, pδCGT and pδYD1, linearized by *Sal*I were transformed into yeast. In comparison to the cells carrying the rDNA in episomal state, cells harboring the *cgt*[3−18] gene in the chromosome showed a larger halo around the colonies when tested using the iodine solution, and the size of the halo was almost equal among positive clones (**Figure 2**). Hydrolysis of starch was examined by reacting equal amounts of the yeast cells carrying either episomal or integrated rDNA with 1% soluble starch in

Table 1. Texture Profile Analysis of Control and *S. cerevisiae*/pδCGT-Treated Bread (**A**) and Rice Cakes (**B**)

(**A**)

properties	control	*S. cerevisiae*/pδCGT
hardness (g)	526.5 (±33.5)	237.9 (±13.3)
cohesiveness	0.45 (±0.05)	0.47 (±0.03)
springiness	0.90 (±0.02)	0.83 (±0.01)
chewiness (g)	212.2 (±31.4)	93.5 (±13.9)

(**B**)

properties	control	*S. cerevisiae*/pδCGT
hardness (g)	23611.5 (±183.6)	8325.9 (±14.4)
cohesiveness	0.51 (±0.06)	0.46 (±0.02)
springiness	0.76 (±0.08)	0.81 (±0.01)
chewiness (g)	9250.2 (±93.4)	3102.5 (±9.6)

50 mM sodium acetate buffer (pH 6.0) at 50 °C using the DNS method. Cells carrying the rDNA in the chromosome showed 3.2 times higher starch-hydrolyzing activity than those carrying the episomal rDNA. This observation strongly suggested that the integration rDNA displayed more CGTase[3−18] than the episomal rDNA. Therefore, *S. cerevisiae*/pδCGT was used for the preparation of bread and rice cakes with *S. cerevisiae*/pδYD1 as a control.

Gene Stability. Stability of the *cgt*[3−18] gene in yeast was investigated by sequential batch cultures of the transformants. CGTase[3−18]-displaying cells were cultured in nonselective YPG medium containing 20 g/L galactose for induction of the *cgt*[3−18] gene under the control of the *GAL1* promoter. Aliquots of the cultures taken during 4 days of growth were diluted and spread on YPGS plates containing 1% soluble starch. Their hydrolyzing activity toward starch was examined by the iodine test (*10*), and the gene stability was defined as the percentage of recombinant cells that maintained hydrolyzing activity on the plate. Almost all integration-type yeast transformants retained starch-hydrolyzing activity after 4 days (**Figure 3**), whereas only 63% of *S. cerevisiae* carrying the episomal rDNA retained the rDNA for the same length of time. The results explained why the yeast cells carrying the episomal rDNA showed little effect on bread baking and low reproducibility. Therefore, the integration-type yeast transformant was used for further study.

pH and Temperature Profiles of *S. cerevisiae*/pδCGT. Immobilized enzymes may exhibit characteristics distinct from those of free enzymes. The stability of an immobilized enzyme is likely to be increased or remain constant during heating or storage, depending on how the new microenvironment affects its tendency to denature. The pH and temperature profiles of *S. cerevisiae*/pδCGT were investigated using the DNS method. The yeast cells with CGTase displayed were used as biocatalysts with 1% soluble starch as a substrate at various temperatures and pHs. CGTase[3−18] displayed on yeast had the same optimal pH and temperature, pH 6.0 and 50 °C, respectively, as the free enzyme (**Figure 4**). However, displayed CGTase[3−18] showed slightly higher activity at temperatures above 60 °C and was more stable at pH below 5 and above 7, which might be caused by immobilization on the yeast cell. The results suggested that the displayed enzyme functions as free CGTase[3−18] in solution and is suitable for baking.

Freeze−Thaw Stability. Generally, dough is stored at low temperatures or frozen in industrial baking. Therefore, viability of yeast during storage is very important. During four cycles of the freeze−thaw process, 98% of *S. cerevisiae*/pδCGT remained viable, which is comparable to the 78% viability of wild-type yeast under the same conditions (**Figure 5**). After freezing, about 90% of the CGTase[3−18] activity produced by the recombinant yeast remained. These results indicated that *S. cerevisiae*/pδCGT has a high potential for industrial application. However, the reason why *S. cerevisiae*/pδCGT showed higher viability than wild-type yeast is not clear.

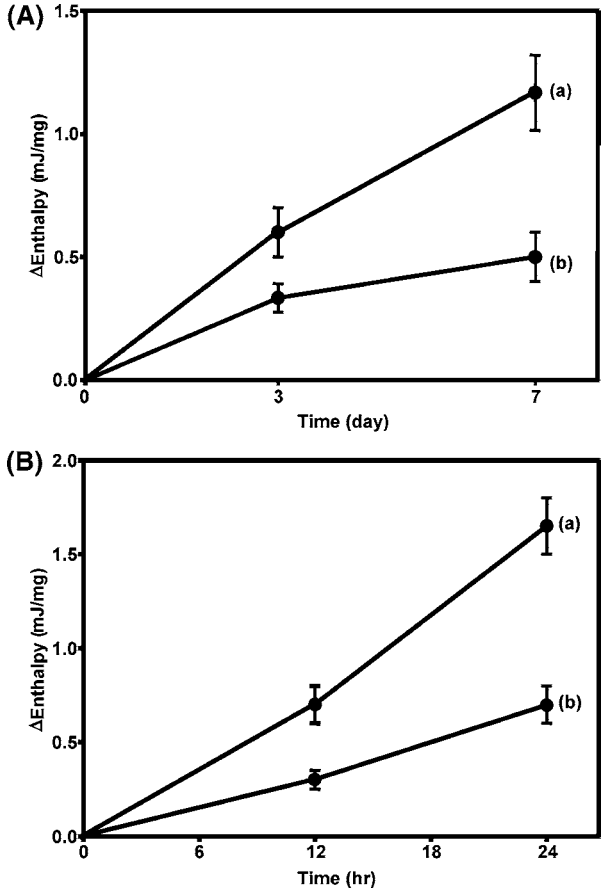

Figure 8. Retrogradation rates of bread loaves (**A**) and rice cakes (**B**) treated with *S. cerevisiae*/pδCGT (**b**) or *S. cerevisiae*/pδYD1 (**a**) during storage.

Volumes after Baking. Volumes of the bread loaves and rice cakes increased by 20% and 45%, respectively, when *S. cerevisiae*/pδCGT was used for baking (**Figure 6**). CGTase[3−18] displayed on the cell surface hydrolyzed starch in bread and rice mixes during baking and steaming, respectively, producing maltooligosaccharides such as glucose and maltose, which can be used in yeast metabolism producing CO_2. This caused an increase in their volumes. Ueda and Tanaka (*22*) reported that yeast could obtain glucose and maltose from the starch medium by the action of displayed amylolytic enzymes on the cell surface.

Production of CDs by CGTase[3−18] displayed on the cell surface was examined by HPLC analysis. No CD production was observed in the bread loaves, suggesting that the engineered yeast is suitable for baking (**Figure 7**). Previously, similar results were obtained using free CGTase[3−18] (*10*).

Texture Analysis after Storage. During storage at 4 °C, physical changes in bread loaves and rice cakes using texture profiles were analyzed (summarized in **Table 1**). Hardness of the bread loaves and rice cakes during storage was caused by retrogradation. The bread and rice cakes baked with the displayed yeast were softer than the controls with *S. cerevisiae*/pδYD1. The degree of bread staling during storage was examined using DSC. The endothermic enthalpy values of bread

and rice cakes prepared using *S. cerevisiae*/pδCGT at the end of the storage period were 0.5 mJ/mg and 0.7 mJ/mg, respectively, which were significantly lower than the control values of 1.6 mJ/mg and 1.2 mJ/mg, respectively (**Figure 8**). The enzyme displayed on the yeast cells effectively retarded retrogradation of the bread and rice cakes.

In conclusion, a CGTase[3−18]-displaying cassette was integrated into the δ-sequence of *S. cerevisiae*, and the enzyme was displayed stably on the yeast cell surface. *S. cerevisiae*/pδCGT showed amylolytic activity toward starch that cannot be utilized by wild-type *S. cerevisiae*. Also, displayed CGTase-[3−18] had an antistaling effect on the bread and rice cake samples. Using the cell surface display method, manufacturers can employ an antistaling enzyme without extra processes for cell culture and enzyme purification. Furthermore, display of the enzyme on the cell surface allows local concentration of the enzyme. Glucose and maltose produced by the enzyme can be transported into the cell efficiently. Accordingly, these results imply that the recombinant *S. cerevisiae*/pδCGT can be used as a baker's yeast with an antistaling effect.

LITERATURE CITED

(1) Nishida, O.; Kuwazaki, S.; Suzuki, C.; Shima, J. Superior molasses assimilation, stress tolerance, and trehalose accumulation of baker's yeast isolated from dried sweet potatoes (hoshi-imo). *Biosci. Biotechnol. Biochem.* **2004**, *68*, 1442−1448.

(2) Randez-Gil, F.; Sanz, P.; Prieto, J. A. Engineering baker's yeast: room for improvement. *Trends Biotechnol.* **1999**, *17*, 237−244.

(3) Shima, J.; Sakata-Tsuda, Y.; Suzuki, Y.; Nakajima, R.; Watanabe, H.; Kawamoto, S.; Takano, H. Disruption of the *CAR1* gene encoding arginase enhances freeze tolerance of the commercial baker's yeast. *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* **2003**, *69*, 715−718.

(4) Van Dijck, P.; Ma, P.; Versele, M.; Gorwa, M. F.; Colombo, S.; Lemaire, K.; Bossi, D.; Loiez, A.; Thevelein, J. M. A baker's yeast mutant (fil1) with a specific, partially inactivating mutation in adenylate cyclase maintains a high stress resistance during active fermentation and growth. *J. Mol. Microbiol. Biotechnol.* **2000**, *2*, 521−530.

(5) Lee, H. W.; Lee, S. H.; Park, K. J.; Kim, J. S.; Kwon, M. H.; Kim, Y. S. Construction and characterization of a pseudo-immune human antibody library using yeast surface display. *Biochem. Biophys. Res. Commun.* **2006**, *346*, 896−903.

(6) Scott, J. K.; Smith, G. P. Searching for peptide ligands with an epitope library. *Science* **1990**, *249*, 386−390.

(7) Auh, J. H.; Lee, S. Y.; Yoo, S. S.; Son, H. J.; Lee, J. W.; Lee, S. J.; Kim, Y. B.; Park, K. H. A novel maltopentaose-producing amylase as a bread antistaling agent. *Food Sci. Biotechnol.* **2005**, *14*, 681−684.

(8) Lee, S. H.; Kim, Y. W.; Lee, S.; Auh, J. H.; Yoo, S. S.; Kim, T. J.; Kim, J. W.; Kim, S. T.; Rho, H. J.; Choi, J. H.; Kim, Y. B.; Park, K. H. Modulation of cyclizing activity and thermostability of cyclodextrin glucanotransferase and its application as an antistaling enzyme. *J. Agric. Food Chem.* **2002**, *50*, 1411−1415.

(9) Dauter, Z.; Dauter, M.; Brzozowski, A. M.; Christensen, S.; Borchert, T. V.; Beier, L.; Wilson, K. S.; Davies, G. J. X-ray structure of Novamyl, the five-domain "maltogenic" α-amylase from *Bacillus stearothermophilus*: maltose and acarbose complexes at 1.7 Å resolution. *Biochemistry* **1999**, *38*, 8385−8392.

(10) Shim, J. H.; Kim, Y. W.; Kim, T. J.; Chae, H. Y.; Park, J. H.; Cha, H.; Kim, J. W.; Kim, Y. R.; Schaefer, T.; Spendler, T.; Moon, T. W.; Park, K. H. Improvement of cyclodextrin glucanotransferase as an antistaling enzyme by error-prone PCR. *Protein Eng., Des. Sel.* **2004**, *17*, 205−211.

(11) Wang, X.; Wang, Z.; Da Silva, N. A. G418 selection and stability of cloned genes integrated at the chromosomal d-integration of *Saccharomyces cerevisiae*. *Biotechnol. Bioeng.* **1996**, *49*, 45−51.

(12) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: a Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989.

(13) Miller, G. L. Use of dinitrosalicylic acid reagent for determination by reducing sugar. *Anal. Chem.* **1959**, *31*, 426−428.

(14) Bourne, M. C. Principles of objective texture measurement. In *Food Texture and Viscosity: Concept and Measurement*; Bourne, M. C., Ed.; Academic Press: New York, 1982; pp 44−117.

(15) Munoz, A. M. Development and application of texture reference scales. *J. Sens. Stud.* **1986**, *1*, 55−83.

(16) Kim, M. D.; Rhee, S. K.; Seo, J. H. Enhanced production of anticoagulant hirudin in recombinant *Saccharomyces cerevisiae* by chromosomal delta-integration. *J. Biotechnol.* **2001**, *85*, 41−48.

(17) Lee, W.; Dasilva, N. A. Application of sequential integration for metabolic engineering of 1,2-propanediol production in yeast. *Metab. Eng.* **2006**, *8*, 58−65.

(18) Dujon, B. The yeast genome project: what did we learn? *Trends Genet.* **1996**, *12*, 263−270.

(19) Romanos, M. A.; Scorer, C. A.; Clare, J. J. Foreign gene expression in yeast: a review. *Yeast* **1992**, *8*, 423−488.

(20) Lee, F. W.; Da Silva, N. A. Sequential delta-integration for the regulated insertion of cloned genes in *Saccharomyces cerevisiae*. *Biotechnol. Prog.* **1997**, *13*, 368−373.

(21) Parekh, R. N.; Shaw, M. R.; Wittrup, K. D. An integrating vector for tunable, high copy, stable integration into the dispersed Ty delta sites of S*accharomyces cerevisiae*. *Biotechnol. Prog.* **1996**, *12*, 16−21.

(22) Ueda, M.; Tanaka, A. Cell surface engineering of yeast: construction of arming yeast with biocatalyst. *J. Biosci. Bioeng.* **2000**, *90*, 125−136.

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