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Modulation of Cyclizing Activity and Thermostability of Cyclodextrin Glucanotransferase and Its Application as an Antistaling Enzyme

SUNG-HO LEE,[†] YOUNG-WAN KIM,[†] SUYONG LEE,[†] JOONG-HYUCK AUH,[†] SUNG-SUK YOO,[‡] TAE-JIP KIM,[†] JUNG-WAN KIM,[§] SUN-TAE KIM,[#] HOE-JIN RHO,[#] JIN-HWAN CHOI,[#] YOUNG-BAE KIM,[⊥] AND KWAN-HWA PARK*,[†]

Research Center for New Bio-Materials in Agriculture and Department of Food Science and Technology, School of Agricultural Biotechnology, Seoul National University, Suwon 441-744, Korea; Department of Culinary Science, Honam University, Kwangju 506-714, Korea; Department of Biology, University of Incheon, Incheon 402-749, Korea; Basic Research Team, Reasearch and Development Center, Tong Yang Confectionery Company, Seoul, Korea; and The Graduate School of Biotechnology, Korea University, Seoul 136-701, Korea

Cyclodextrin glucanotransferase from *Bacillus stearothermophilus* ET1 (CGTase ET1) is a potential antistaling enzyme with cyclodextrin (CD)-forming activity. To reduce cyclization activity of CGTase ET1, phenylalanine residues at 191 and 255 were replaced with a glycine (F191G-CGTase ET1) and an isoleucine (F255I-CGTase ET1), respectively. Temperature optima of both mutant enzymes were lower than that of the wild-type. Cyclization activities of both mutants decreased dramatically, but F255I-CGTase ET1 showed a 2-fold higher hydrolytic activity than the wild-type enzyme. CD content of bread loaf treated with F191G-CGTase ET1 was 28.6% of that treated with wild-type, whereas no CD was detected in the loaf treated with F255I-CGTase ET1. Loaves treated with CGTase ET1 or either of the two mutants contained more of the larger maltooligosaccharides such as maltopentaose and maltohexaose than the control and the commercial antistaling enzyme-treated loaves. Retrogradation rates decreased significantly in the loaves treated with either mutant, which indicates the applicability of CGTase ET1 in the bread industry by modulating the cyclizing and hydrolyzing activities of the enzyme.

KEYWORDS: Cyclodextrin glucanotransferase (CGTase); retrogradation; antistaling enzyme; cyclization; site-directed mutagenesis

INTRODUCTION

Various undesirable changes occur in bread during storage, among which retrogradation deteriorates the quality of the bread, thereby lowering the consumers' acceptance. However, the exact mechanism of bread retrogradation remains under debate. Various enzymes, emulsifiers, oligosaccharides, and polysaccharides have been used to retard the deterioration process (*1*). Application of enzymes has been investigated for several years, and in fact enzymes are currently used by the bread industry to meet consumers' preference for natural products.

Effective antistaling enzymes such as amylases hydrolyze amylose and amylopectin chains into smaller fractions, which lead to less crystallization as well as limitation of the crystal size (*2*). Low molecular weight dextrins produced by antistaling enzymes decrease the retrogradation rate of bread by inhibiting the interaction between starch and continuous protein matrix (*3*). Moreover, short amylopectin chains enhance the formation of amylopectin−lipid complexes, which consequently delay the retrogradation of bread (*4*). Enzymes with intermediate temperature stability (ITS) have been recommended as effective antistaling agents in bread preparation due to their optimal activities above the gelatinization temperature of starch and complete inactivation of activities at the baking temperature (*5*). Recently, Min et al. (*6*) reported on the usage of G2- and G4-producing amylases with ITS as antistaling agents for bread.

A thermostable CGTase isolated from *Bacillus stearothermophilus* ET1 (CGTase ET1) with a temperature optimum at 80 °C produces CDs as well as various maltooligosaccharides (*7*), making the enzyme a potential antistaling agent for bread. Mutsaers et al. (*8*) reported that the loaf volume increased due to CD produced by CGTase added into the bread dough. However, no report has been documented on CGTase as an antistaling agent retarding the retrogradation of bread. Use of

* Author to whom correspondence should be addressed (telphone 82-31-290-2582; fax 82-31-293-4789; e-mail parkkh@plaza.snu.ac.kr).
[†] Seoul National University.
[‡] Honam University.
[§] University of Incheon.
[#] Tong Yang Confectionery Co.
[⊥] Korea University.

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CDs produced by the action of CGTase in bread could cause difficulties in many countries where CDs are not allowed as food additives. Therefore, it became desirable to develop mutant CGTases that produce high amounts of maltooligosaccharides but no CD. Three-dimensional structure analysis and site-directed mutagenesis of CGTase have been carried out to understand the structure and functional relationship of the enzyme (*9*−*13*). In a previous investigation, increase in the saccharifying activity of CGTase was observed when the conserved hydrophobic aromatic residues involved in the cyclization were replaced with other amino acid residues (*14*, *15*).

The purpose of this study was thus to engineer CGTase ET1, such that the enzyme produced no or fewer CDs similar to other antistaling enzymes, by replacing the aromatic residues at the positions essential for efficient cyclization. The resulting enzymes were also evaluated as antistaling agents for bread.

MATERIALS AND METHODS

Site-Directed Mutagenesis of CGTase Gene. The CGTase gene isolated from *B. stearothermophilus* ET1 (*7*) was mutagenized using a Quikchange site-directed mutagenesis kit (Stratagen), and the mutations introduced were confirmed through automatic sequencing using a BigDye Terminator Cycle sequencing kit for the ABI377 PRISM sequencer (Perkin-Elmer). In the mutant F191G-CGTase ET1, the phenylalanine residue at 191 (F191) was substituted with a glycine using two synthetic oligonucleotides, 5′-TATCGGAATTTG**GGT**-GATTTGGCAG-3′ (F191GN) and 5′-CTGCC AAATC**ACC**CAAAT-TCCGATA-3′ (F191GC). Another set of synthetic oligonucleotides, 5′-GGGGAATGG**ATT**TTGTCAGAAAATG-3′ (F255IN) and 5′-CATTTTCTGACAA**AAT**CCATTCCCC-3′(F255IC), was used to replace F255 with an isoleucine. The nucleotides coding the mutated amino acid residues are shown in bold letters. Other genetic manipulations were carried out according to the methods described by Sambrook et al. (*16*).

Purification of Wild-Type and Mutant CGTases. The wild-type gene encoding CGTase ET1 and those for two mutant enzymes obtained by site-directed mutagenesis were subcloned on pUB140, a *Bacillus* vector, and expressed in *Bacillus subtilis* LKS88 (*17*). The transformants carrying the wild-type and mutated CGTase genes were cultured in LB medium containing 1% (w/v) soluble starch (Showa) and 10 μg/mL kanamycin (Sigma) for 24 h at 37 °C with vigorous shaking. The enzymes were purified by β-CD affinity column chromatography as described previously by Chung et al. (*7*). Active fractions were concentrated through ultrafiltration (Amicon Co.) and then dialyzed against 50 mM maleate−NaOH buffer (pH 6.0).

Enzyme Assay. Hydrolytic activity of CGTase was assayed according to the method described by Kim et al. (*18*) with a minor modification. Enzyme solution (0.05 mL) diluted in 0.2 mL of 50 mM maleate−NaOH buffer (pH 6.0) was added to prewarmed 1% soluble starch (Showa) dissolved in the same buffer (0.25 mL) and incubated at the optimal temperature of each enzyme for 10 min. The reaction was stopped by adding 0.5 mL of 3,4-dinitrosalicylic acid (*19*) and boiled for 5 min for color development. The mixture was then cooled immediately under running tap water, and the absorbance of the mixture was measured at 575 nm. One unit of soluble starch hydrolyzing activity (SU) was defined as the amount of enzyme required to produce reducing sugar equivalent to one unit change of absorbance at 575 nm. The cyclization activity of CGTases was assayed as described by Kaneko et al. (*20*) with some modification. The enzyme solution (20 μL) was added into 1 mL of 4% (w/v) gelatinized soluble starch dissolved in 50 mM maleate−NaOH buffer (pH 6.0) and incubated at the optimal temperature of each enzyme for 10 min. The reaction was stopped by adding 3.5 mL of 30 mM NaOH. Subsequently, 0.5 mL of phenolphthalein (0.02% in 5 mM Na_2CO_3) was added to the reaction mixture, which was then left standing at room temperature for 15 min. Absorbance of the reaction mixture was measured at 550 nm, and the concentration of β-CD was determined by extrapolating β-CD to a standard curve. One unit of CD-forming activity (CFU) was defined as the amount of enzyme required to produce 1 mg of β-CD per minute. Protein concentration was measured using the Bradford method (*21*) with bovine serum albumin as the standard.

Conditions for Bread Baking. Bread loaves were baked under five different conditions using White Pan Bread Mix II (Cheiljedang Co.) in an automatic home breadmaker (SHB-200, Samsung Co.) and were stored at 4 °C in polyethylene bags. The formulation of the bread mix was as follows: 100 parts of white flour, 8.9 parts of sugar, 5.2 parts of shortening, 2.2 parts of defatted milk powder, 0.1 part of yeast, 1.4 parts of salt, and 69 parts of water (parts by weight). Five setups for preparing bread were as follows: control using the bread mix alone; bread mix supplemented with 500 SU of wild-type CGTase; bread mix supplemented with 210 SU of the mutant F191G-CGTase ET1; bread mix supplemented with 200 SU of the mutant F255I-CGTase ET1; and bread mix supplemented with 0.02% (w/w, 7000 SU) Novamyl (Novo Nordisk).

Differential Scanning Calorimetry (DSC). The retrogradation rate of each loaf was determined through DSC using a DSC 120 (Seiko Co.) calibrated with indium and tin on days 3 and 7 during the storage of bread at 4 °C as described by Kweon et al. (*4*). Distilled water was used as a reference. Bread samples (10 mg) were weighed and hermetically sealed in aluminum pans. The pans were then heated from 20 to 130 °C at 5 °C/min. The degree of retrogradation was expressed as the enthalpy calculated from the area of endothermic peak between 40 and 80 °C.

Analysis of Maltooligosaccharides and CD. A mixture of 10 g of bread crumb and 100 mL of distilled water was stirred vigorously for 1 h and then centrifuged (10000 rpm, 20 min). The supernatant (25 mL) was concentrated by drying in a freeze-dryer (Ilshin Engineering Co.) and then resuspending the pellet in 5 mL of distilled water. The composition of maltooligosaccharides in each sample was analyzed through high-performance anion exchange chromatography (HPAEC) using a CarboPac PA1 column (0.4 × 25 cm, Dionex) and an electrochemical detector (ECD40, Dionex). The sample was filtered through a membrane filter (0.2 μm pore diameter, Gelman Sciences) and then injected into the column. Two buffers, A (150 mM NaOH in water) and B (600 mM sodium acetate in buffer A), were used for elution of the sugars with 0−30% (v/v) gradient of buffer B at a flow rate of 1.0 mL/min. To measure the amount of CDs in each bread sample, the phenolphthalein method described above was used.

RESULTS AND DISCUSSION

Cyclizing and Hydrolysis Activities of Wild-Type and Mutant CGTases. Amino acid sequences around the aromatic residues essential for the cyclization activity of various CGTases were aligned as shown in **Figure 1**. Nakamura et al. (*22*) reported that four aromatic residues at the active center were involved in the cyclization activity of CGTase from *Bacillus* sp. 1011. Among the residues, Y195 located in the hydrophobic core was known to stabilize the spiral inclusion complex with amylose. F259, localized outside the circular structure, stabilized the intermediate complex of the cyclization reaction by interacting with glucose (*23*). These residues are equivalent to F191 and F255 of CGTase ET1.

Phenylalanine residues at positions 191 and 255 were replaced with a glycine and an isoleucine, and the resulting mutants were designated F191G-CGTase ET1 and F255I-CGTase ET1, respectively. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) verified that they were homogeneous (data not shown), and their molecular weights were similar to that of the wild-type (66800 Da). The optimal temperatures of F191G-CGTase ET1 and F255I-CGTase ET1 were 60 and 75 °C, respectively, whereas that of the wild-type was 80 °C (**Figure 2**). Lower optimal temperatures of the mutant enzymes made them more suitable as antistaling agents with ITS. In general, enzymes with optimal temperatures in the range of 50−60 °C are considered to be ITS enzymes.

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                                                    191
CGTET1      SYFHHNGG----TTFSNLED-GIY--R--NLFDLADFNHQN
BCIR251     NLFHHNGG----TDFSTTEN-GIY--K--NLYDLADLNHNN
BSNO2       MYFHHNGG----TTFSSLED-G-Y--R--NLFDLADLNHQN
BAC1011     NLFHHYGG----TDFSTIEN-GIY--K--NLYDLADLNHNN
BLCGT       GYFHHNGG----SDFSTLEN-GIY--K--NLYDLADLNHNN
BMCGT       GLFHHNGG----TDFSTIED-GIY--K--NLYDLADINHNN
KLEB        GWYHHNGGVTNWNDFFQVKNHNIFNLKNHNIFNLSDLNQSN
                          *                 *

                255
CGTET1      FGEWFLSENEVDSNNH-----FFANESGMS-LLDFRFGQKL
BCIR251     FGEWFLGVNEVSPENH-----KFANESGMS-LLDFRFAQKV
BSNO2       FGEWFLSENEVDANNH-----YFANESGMS-LLDFRFGQKL
BACCGT3     FGEWFLGVNEISPEYH-----QFANESGMS-LLDFPFAQKA
BLCGT       FGEWFLGSAAPDADNT-----DFANESGMS-LLDFRFNSAV
BMCGT       FGEWYLGADQTDGDNI-----KFANESGMN-LLDFEYAQEV
KLEB        FGEWF-GASANTTTGVDGNAIDYANTSG-SALLDFGFRDTL
                  *                       *
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Figure 1. Alignment of conserved aromatic residues essential for cyclization activity of various CGTases. Four aromatic residues essential for the cyclization activity are marked with asterisks. Two amino acids (F191 and F255) that were mutagenized in this study are shown in black boxes and boldfaced. CGTET1 is CGTase from *B. stearothermophilus* ET1, BCIR251 is CGTase from *B. circulans* 251, BSNO2 is CGTase from *B. stearothermophilus* NO2, BLCGT is CGTase from *B. licheniformis*, BMCGT is CGTase from *B. macerans*, and KLEB is CGTase from *Klebsiella pneumoniae*.

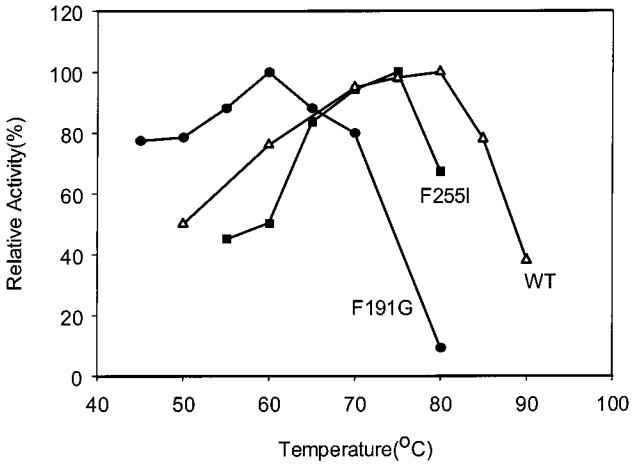

Figure 2. Optimal temperatures of wild-type and mutant CGTases: effect of temperature on (△) wild-type CGTase ET1, (●) F191G-CGTase ET1, and (■) F255I-CGTase ET1.

Table 1. Comparison of the Properties of Wild-Type and Mutant CGTases

enzyme	optimal temp (°C)	cyclizing activity (CFU/mg)	hydrolyzing activity (SU/mg)	ratio H/C[a]
WT	80	192.9 ± 31.1	45.6 ± 6.5	0.2
F191G	60	27.1 ± 2.8	21.3 ± 1.7	0.8
F255I	75	19.0 ± 1.7	101.1 ± 5.2	5.3

[a] Hydrolyzing activity/cyclizing activity.

Table 1 shows the comparison of cyclization and hydrolysis activities of the wild-type and two mutant CGTases. Cyclization activities of F191G-CGTase ET1 and F255I-CGTase ET1 decreased significantly to 14.0 and 9.8%, respectively, of the wild-type activity. F191G-CGTase ET1 retained 46.7% hydrolytic activity of the wild-type, whereas the hydrolytic activity of F255I-CGTase ET1 increased by 2-fold compared to the wild-type. These results suggested that phenylalanines at 191 and 255 might constitute the active site and are likely to be involved in the cyclization reaction, synthesizing CDs from starch.

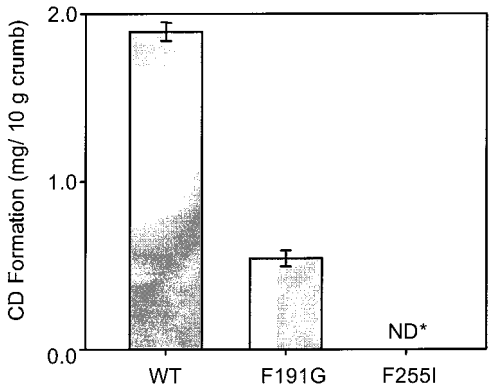

Figure 3. Amount of CDs in loaves treated with various CGTases. ND indicates that no CD was detected.

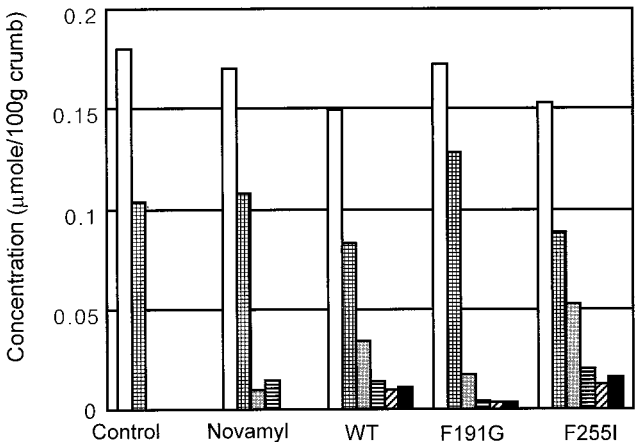

Figure 4. Composition of maltooligosaccharides in loaves treated with various CGTases: (white bars) amount of glucose; (hatched bars) maltose; (gray bars) maltotriose; (lined bars) maltotetraose; (slashed bars) maltopentaose; (black bars) maltohexaose.

Production of CDs by CGTases. The amount of CDs produced in bread by each CGTase enzyme was analyzed (**Figure 3**). The loaves treated with the mutant CGTases had much less CD than the bread treated with the wild-type enzyme. The amount of CD produced by F191G-CGTase ET1 was only 26.9% of that produced by the wild-type enzyme, whereas the amount of CD in the bread treated with F255I-CGTase ET1 was too little to be quantified. Because the hydrolytic activity of F255I-CGTase ET1 was much higher than that of F191G-CGTase ET1, the former was added to bread at an amount 5 times less than the latter. This led to the production of almost no CD, whereas 0.54 mg of CD was produced in 10 g of F191G-CGTase ET1-added bread.

Production of Maltooligosaccharides by CGTases in the Loaves. Various maltooligosaccharides produced in the loaves were analyzed via HPAEC (**Figure 4**). The composition and amount of maltooligosaccharides in the loaves varied depending on the enzyme treated. In all enzyme-added loaves, the major products were maltose, maltotriose, maltotetraose, and maltopentaose. Maltotriose, maltotetraose, and maltopentaose are significantly more hygroscopic than other maltooligosaccharides, whereas the hygroscopicity of maltose is the lowest (*24*). Novamyl, a commercial antistaling enzyme, produced mainly maltotriose and maltotetraose (24 nmol/100 g of crumb). In addition, the loaves treated with CGTases contained maltopentaose and maltohexaose, which were not detected in those

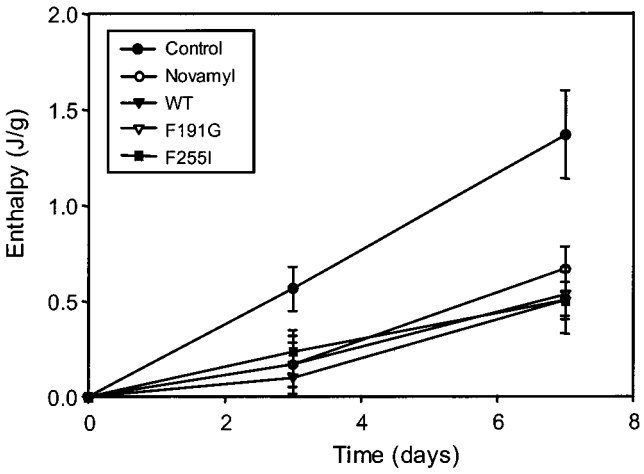

Figure 5. Retrogradation rates of loaves treated with various enzymes during storage: change of enthalpy in the (●) control loaf (no enzyme treated), (○) Novamyl-treated loaf, (▼) wild-type CGTase-treated loaf, (▽) F191G-CGTase-treated loaf, and (■) F255I-CGTase-treated loaf.

treated with Novamyl, probably due to the disproportionation activity of CGTase. In particular, in the loaf treated with F255I-CGTase ET1, the content of maltooligosaccharides that were longer than maltose was higher than the others (105 nmol/100 g of crumb), an indication that the enzyme would make the most effective antistaling agent that reduces the retrogradation rate of bread. The high hygroscopicity of many maltooligosaccharides produced by CGTases would interfere with the starch−starch interactions that cause retrogradation by holding water around the starch. In addition, CGTases hydrolyzed starch very efficiently, and the molecular weights of amylopectin and amylose decreased significantly (data not shown). Consequently, smaller starch fractions would not favorably interact with each other to form crystals, thereby slowing the retrogradation rate of bread.

Analysis of Bread Retrogradation. Generally, the first endothermic peak in the curve of thermal analysis appears at ∼40−80 °C. This staling endotherm results from the melting of crystalline amylopectin when starch solution or starchy food is heated (*4*, *25*). DSC analysis, therefore, enables the measurement of the retrogradation degree of bread during storage because amylopectin is considered to be the main component responsible for bread retrogradation. Throughout the storage period, retrogradation rates of the experimental loaves were much lower than that of the control loaf (**Figure 5**). This result was in good correlation with the data obtained from the analysis of maltooligosaccharide composition in the loaves. Moreover, the mutant CGTase was as effective as Novamyl in retarding the retrogradation of bread. The volume of bread has been reported to increase due to the production of CD upon adding CGTase (*8*). However, volumes of the loaves treated with enzymes increased compared to that of the control loaf (data not shown), even when a CGTase mutant that did not produce CD was added.

In conclusion, the two CGTase mutants were characterized by intermediate temperature stability and the production of high amounts of maltooligosaccharides with significantly reduced or no detectable CD. The retrogradation rates of the bread treated with the mutant CGTases were much lower than that of the control bread, and the efficiencies of the mutant CGTases were comparable to that of Novamyl. These results provide strong indications of their possibilities of being used as effective antistaling enzymes.

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