Improvement of cyclodextrin glucanotransferase as an antistaling enzyme by error-prone PCR

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In an effort to improve the properties of cyclodextrin glucanotransferase (CGTase) as an antistaling enzyme, error-prone PCR was used to introduce random mutations into a CGTase cloned from alkalophilic Bacillus sp. 1-5 (CGTase I-5). A mutant CGTase[3-18] with the three mutations M234T, F259I and V591A was selected by agar plate assay. Sequence alignment of various CGTases indicated that M234 and F259 are located in the vicinity of the catalytic sites of the enzyme and V591 in the starch binding domain E. The cyclization activity of CGTase[3-18] was dramatically decreased by 10-fold, while the hydrolyzing activity was increased by up to 15-fold. These mutations near subsite +1 (M234T) and at subsite +2 (F259I) are likely to alter the enzyme activity in a concerted manner, promoting hydrolysis of substrate while retarding cyclization. The addition of CGTase[3-18] reduced the retrogradation rate of bread by as much as did the commercial antistaling enzyme Novamyl during 7-day storage at 4°C. No cyclodextrin (CD) was detected in bread treated with wild-type CGTase[3-18], whereas 21 mg of CD per 10 g of bread was produced in bread treated with wild-type CGTase.

Keywords: antistaling enzyme/CGTase/error-prone PCR/random mutagenesis/retrogradation

Introduction

After bread is baked, a series of physicochemical changes develop in the loaf that lead to deterioration of quality during storage. A change in crumb firmness, called retrogradation, is one of the major factors affecting consumer acceptance. Therefore, several additives such as chemicals, small sugars and enzymes have been included in bread mix in efforts to preserve the softness of bread for longer storage periods.

α-Amylases have an antistaling effect on bread because they retard retrogradation of the loaf (De Stefanis and Turner, 1981; Sahlström and Bråthen, 1997). Effective antistaling enzymes, such as amylases, hydrolyze amylose and amylopectin chains into smaller molecules, leading to less crystallization and limited crystal size (Boyle and Hebeda, 1990). Low molecular weight dextrins produced by antistaling enzymes decrease the retrogradation rate of bread by inhibiting the interaction between starch and the continuous protein matrix (Martin and Hoseney, 1991). In addition, shorter amylase chains form more complexes of amylpectin and lipid, consequently retarding retrogradation of bread (Kweon et al., 1994). Leon et al. (2002) investigated the effect of amylase–lipase mixtures on retrogradation of bread. They reported that maltose and water-soluble dextrin (DP 4–6) were most effective in preserving crumb softness. However, the use of α-amylase has become limited despite its antistaling effect because too much of the enzyme can cause stickiness in bread. De Stefanis and Turner (1981) explained that it is the production of branched maltooligosaccharides of DP 20–100 by α-amylase that leads to gumminess in bread. To solve the problem, Carroll et al. (1987) used a mixture of α-amylase and pullulanase in bread baking. Pullulanase hydrolyzes the branched maltoligosaccharides of DP 20–100 produced by the α-amylase into smaller maltolloigosaccharides, which improves the quality of baked goods (Kim et al., 2000). Min et al. (1998) employed an amylase that produces mainly maltooligosaccharides as an antistaling agent for bread baking. The maltotriose and maltotetrose produced by this enzyme seem to retard retrogradation of bread. Cyclodextrin glucanotransferases (CGTases; EC 2.4.1.19) 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. Owing to this restriction, the development of mutant CGTases that produce large amounts of maltoligosaccharides but no CD has been attempted. The analysis of tertiary structure and site-directed mutagenesis of CGTase have been carried out in order to understand the structure–function relationship (Fujiwara et al., 1992a; Lawson et al., 1994; Penninga et al., 1996; Beier et al., 2002; Shin et al., 2000). The results revealed that substitution of the conserved hydrophobic aromatic residues involved in cyclization of CGTase leads to an increase in the saccharifying activity of the enzyme (Fujiwara et al., 1992b; Leemhuis et al., 2002). Recently, we reported the potential adaptation of CGTase from Bacillus stearothermophilus ET1 as an antistaling agent for bread by eliminating the cyclization activity of the enzyme via site-directed mutagenesis at F191 and F255 (Lee et al., 2002). However, to improve the properties of enzymes, the site-directed mutagenesis limit needs to be overcome.

CGTase I-5 was isolated from an alkalophilic Bacillus sp. and had an optimal temperature and pH of 60°C and 6.0, respectively. It produced mainly β-CD from starch. The purpose of this study was to engineer CGTase I-5 by random mutagenesis using error-prone polymerase chain reaction...
(PCR) such that the enzyme produced no CD or less CD than is produced by other antistaling enzymes. CGTase I-5 was used for this purpose, because substantial amounts of this enzyme can be purified easily via DNA manipulation of the structural gene. The enzymatic properties of the engineered enzymes were analyzed and the enzymes were also evaluated as antistaling agents for bread.

Materials and methods

Bacterial strains

Escherichia coli MC1061 [F-, araD139, recA13, Δ(araABC-leu)7696, galU, galK, ΔlacX74, rpsL, thi, hsdR2, mcrB] was used as a host for DNA manipulation and transformation. Escherichia coli MC1061 was grown in LB medium [1% (w/v) Bacto-tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl]. Escherichia coli transformants were grown in LB medium supplemented with ampicillin (100 μg/ml). An alkalophilic Bacillus sp. strain I-5 was isolated from Korean soil in our laboratory and used as the source of the CGTase gene (cgtI5; GenBank accession No. AY478421) in this study.

Construction of pR2CGT

The cgtI5 gene was cloned in pBR322 and the resulting construction was designated as pCGTJ322. The 2.5 kb SalI−HindIII fragment containing cgtI5 was subcloned into pR2BS, an expression vector, by placing the gene under the control of the amyR2 promoter on the vector to enhance the expression level of the gene product (Figure 1). The resulting recombinant DNA, pR2CGT, was transformed into E. coli MC1061 as described by Sambrook et al. (1989) and used for DNA manipulation and enzyme production.

Error-prone PCR

Random mutagenesis of cgtI5 was carried out by error-prone PCR (Song and Rhee, 2000). Two synthetic oligonucleotides, CGT1−5N (5'-CCCTAAGCTTGTGAGCGTGTTAGGAGCTGGATA-GTATG-3') and CGT1−5C (5'-CCCTAAGCTTGTGAGCGTGTTAGGAGCTGGATA-GTATG-3'), were designed to have a SalI and a HindIII restriction site (underlined), respectively. PCR conditions for random mutagenesis were optimized such that mutation at a desirable level (three or four amino acid substitutions) and without bias in base substitution could be achieved. The PCR reaction mixture (100 μl) consisted of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 7 mM MgCl₂, 0.1 mM MnCl₂, 0.2 mM dATP, 0.2 mM dGTP, 1 mM dCTP, 1 mM dTTP, 25 pmol of each oligonucleotide primer, 5 ng of template DNA (pR2CGT) and 5 U of Taq polymerase (Takara Shuzo, Japan). PCR was performed in an automatic thermal cycler (GeneAmp 9600, Perkin-Elmer, USA) for 30 cycles, each of which consisted of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min.

The resulting mutant PCR products were digested with SalI and HindIII restriction enzymes and then ligated into the corresponding sites on pR2BS. The ligation mixture was transformed into E. coli MC1061 and spread on LB agar plates containing ampicillin (100 μg/ml). Three rounds of PCR were carried out using a mutant selected from the previous step as a template. The mutants were screened as described below.

Site-directed mutagenesis

To check the effects of the mutation at each of the three sites (M234T, F259I and V591A), the CGTase I-5 gene was mutated using a QuikChange site-directed mutagenesis kit (Stratagene, USA). The primers M234T-N (5'-CGCGGTCAAGCATACG-

CCATCGCGGTCGCGACG-3') and M234T-C (5'-CTGCCATGCCGGTAGTATGACTTGACG-3') were used to construct the M234T mutant. For the F259I mutation, the primers F259I-N (5'-AACCCGGTGTCACCCATGGGAGATCG-3') and F259I-C (5'-AAGGAACCATCTGGCGCAATGCCGGTGGGAGATCG-3') were used. Another set of primers, V591A-N (5'-GTCACCGTGTGCATCGAATCCAAAATGCCGAG-3') and V591A-C (5'-GACGCTACGAGATCGAATCCAAAATGCCGAG-3') was used to replace a valine at 591 with an alanine. The underlined nucleotides correspond to the desired substitution of amino acid residues. All mutations were confirmed by dideoxy chain-termination sequencing using an ABI377 PRISM DNA sequencer (Perkin-Elmer).

Screening of the mutants

Escherichia coli MC1061 transformants carrying mutant CGTases with enhanced hydrolyzing activity but no cyclization activity were screened by the iodine and phenolphthalein tests using LBS agar plates [LB broth containing 1% (w/v) soluble starch, 1.5% (w/v) agar and 100 μg/ml ampicillin], in terms of the size of clear zone and color change around colonies, respectively. After 10 h of incubation on LBS plates at 37°C, 10 ml of iodine solution (0.203 g I₂, 5.2 g KI, distilled H₂O to 100 ml) or 5 ml of phenolphthalein top agar [2% (w/v) Na₂CO₃, 0.6% (w/v) agar, 0.1% (w/v) K₂HPO₄, 0.02% (w/v) phenolphthalein, 0.02% (w/v) methyl orange] were overlaid on the plates (Park et al., 1989). About 1000 colonies from each round were selected primarily based on the size of clear zone formed around the colony in the iodine test. Among them, 100 colonies with a smaller orange-colored zone than that of wild-type in the phenolphthalein test were selected for further analyses. The activities of 100 positive colonies from each round were assayed using their crude cell extracts; 20 mutants from each round with appropriate activity were selected.
purified and analyzed further. DNA sequencing of the genes for selected mutants was carried out as described above to confirm the introduced mutations.

**Purification of CGTases**

*Escherichia coli* MC1061 carrying the wild-type or mutant CGTase clone was cultured in 100 ml of LB broth at 37°C for 12 h in a 500 ml baffled flask with shaking at 200 r.p.m. The cells were then transferred to a 5 l jar fermenter (KFC, Korea) containing 3 l of LB broth and were cultured at 37°C for 24 h with sufficient aeration and agitation at 200 r.p.m. The cells were harvested by centrifugation at 4°C, resuspended in 300 ml of 50 mM sodium acetate buffer (pH 6.0) and sonicated in an ice-bath using a sonicator (VC-600, Sonics and Materials, USA) (output 4–5 min×3 times, 60% duty). The enzyme in the crude cell extract was purified using a β-CD affinity column as described by Chung et al. (1998). Active fractions were collected, concentrated by ultrafiltration (Amicon, USA) and dialyzed against 50 mM sodium acetate buffer (pH 6.0). Protein concentration was determined by the Bradford method (Bradford, 1976) with bovine serum albumin as the standard. The yield of the recombinant enzymes was about 5 mg/l of culture. The purity and molecular weight of the purified proteins were analyzed by 10% SDS–PAGE.

**Enzyme assay**

The hydrolytic activity of CGTase was measured by the DNS method (Miller, 1959). A mixture of 0.25 ml of 1% (w/v) soluble starch (average molecular weight 1×10⁵ g/mol) dissolved in 50 mM sodium acetate buffer (pH 6.0) and 0.2 ml of the same buffer was pre-warmed at 60°C for 5 min. CGTase (1 μg for mutant, 10 μg for wild-type) was added to the solution and the reaction was carried out at 60°C for 10 min. The reaction was stopped by the addition of 0.5 ml of 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. The absorbance of the solution was measured at 575 nm (Ultrospec III, Pharmacia, Sweden). The blank was run in the same way but without the enzyme. One unit (U) of the hydrolyzing activity was defined as the amount of enzyme that split 1 μmol equivalent of glycosidic bond in soluble starch per minute. The cyclization activity of CGTase was assayed by the phenolphthalein method (Kaneko et al., 1991). The kinetics of cyclization activity were measured by determining the β-CD forming activity of the enzyme (0.3–3 μg) using the phenolphthalein method. The soluble starch concentrations used in the determination of kinetic parameters were 0.5–5 and 0.5–10 times the Kₘ values for the hydrolyzing and the cyclization activity, respectively. The kinetic parameters were determined by fitting a Lineweaver–Burk plot with the SigmaPlot program (version 5.0; SPSS, Chicago, IL).

**Structure modeling**

The three-dimensional structure of CGTase[3–18] was modeled using the SWISS-MODEL version 3.51 program at the ExPASy server. The structures of various CGTases reported previously were used as modeling templates. The RCSB Protein Data Bank entries for these proteins are 1A47, 2DJ and 1D7F. The modeled structure was visualized and analyzed using the Swiss-PdbViewer version 3.51. The figures were created with MOLSCRIPT version 2.1.

**Bread baking**

All four groups of bread loaves were baked with White Pan Bread Mix II (Cheil Jedang, Korea) as described previously (Lee et al., 2002). Bread loaves were baked in the following four set-ups: control using the bread mix alone; bread mix supplemented with 0.02% (w/w) Novamyl (~2000 U); bread mix supplemented with 0.012% (w/w) wild-type CGTase (120 U); and bread mix supplemented with 0.001% (w/w) CGTase[3–18] (120 U). Bread loaves were stored at 4°C in polyethylene bags. Novamyl 1500MG, an antistaling amylase, was kindly donated by Novozyme (Korea). The volume of each bread loaf was measured by the rice grain displacement procedure.

**Analysis of CD in bread**

Bread crumbs (10 g) were extracted with 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 (Gelman Sciences, USA). A 25 ml volume of the supernatant was dried using a freeze-dryer (Ishin Engineering, Korea) and then dissolved in 5 ml of water. The concentration of CD in the bread was analyzed using the phenolphthalein method as described above. HPLC was also carried out to measure the CD present in the loaves. The HPLC system used in this study (SLC-100, Samsung Electronics, Korea) was equipped with a LiChrosorb NH₂ column (10 μm, 4×250 mm) (Merck, Germany) and a differential refractometer (400R, Waters, USA). The samples were eluted at a flow rate of 1.0 ml/min using a mixture of acetonitrile and water (65:35, v/v).

**Differential scanning calorimetry (DSC)**

The retrogradation rate of each loaf was determined through DSC using a DSC 120 instrument (Seiko, Japan) at days 3 and 7 during the storage of bread at 4°C (Kwone et al., 1994). 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. Bread 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.
The enzyme (Figure 2). M234 is located adjacent to the and F259 are located in the vicinity of catalytic sites of Sequence alignment of different CGTases indicated that M234 L210V, L551P, R553A and V639I, appeared to be neutral. Mutations at other sites, such as G35S, D39N, A101G, another 2-fold and led to a 10-fold reduction in cyclization activity. However, the introduction of F259I in the final round a further 3-fold without a significant decrease in cyclization activity. M234T in CGTase[2±28] increased the hydrolyzing activity slightly in CGTase[1±7]. The additional introduction of V591A into wild-type CGTase I-5 increased the hydrolyzing activity of CGTase has been reported previously (Fujiwara et al., 1999; van der Veen et al., 2001). Based on the predicted tertiary structure, T234 is not likely to be located within a distance that could affect substrate binding, but it could cause a change in the loop in which A230, K232 and H233, the residues interacting with the +1 and +2 subsites, are located (Figure 3). According to a recent mutagenesis study of B.circulans 251 CGTase, the residue A230 at subsite +1 plays an important role in the cyclization activity of CGTase (Leemhuis et al., 2003). The authors proposed that the A230V mutation would negatively affect cyclization activity by steric hindrance at subsite +1. Hence the M234T mutation from a bulky amino acid (Met) to a smaller one (Thr) might lead to a change in the position of the peptide backbone near the mutation and could thus alter the torsional angles of A230, K232 and H233. Conclusively, these results suggest that the mutations near the subsite +1 (M234T) and at subsite +2 (F259I) alter enzyme activities in a concerted manner, promoting hydrolysis of substrate while retarding cyclization. V591 is located in domain E, which is known as the starch-binding domain (Svensson et al., 1989; Figure 2). At this point, however, no speculation could be provided on how the mutation contributed to the resulting properties. The residue is not only far from the active site but also not closely located to the two maltose-binding sites in the E-domain. Based on the data obtained, CGTase[3±18] was examined further for its possible use as an antistaling agent.

Characterization of mutant CGTases

The wild-type and mutant CGTase I-5 enzymes were purified using a β-CD column and the purity of the enzymes was verified by SDS–PAGE (data not shown). The optimal temperatures for the hydrolyzing activity of wild-type CGTase and CGTase[3±18] were 60 and 50°C, respectively (Table II). The optimal pH for the hydrolyzing activity of wild-type and mutant CGTase I-5 was 6.0, even though the wild-type was more stable than the mutant enzyme at acidic (pH 4–5) and alkaline (pH 8–10) pHs (data not shown). The mutant enzyme was as stable as the wild-type at the optimal pH.

Results and discussion

Construction of mutant CGTases

The 2.5 kb Salt–HindIII fragment of pR2CGT carrying the cgf5 gene from alkalophilic Bacillus sp. strain I-5 was isolated and used as a template for the construction of mutants by error-prone PCR. Six mutants with the most appropriate phenotypes were finally selected and their genetic and enzymatic characteristics were analyzed as summarized in Table I. CGTase[1–7] was used as the template for the second round of PCR and CGTase[2–5], [2–28], [2–32] and [2–40] for the third round. Nucleotide sequencing analysis of the mutations introduced into the enzyme indicated that mild random mutation had accumulated in the mutant obtained from the final round during the sequential error-prone PCR processes. Among the mutants obtained from the final round, CGTase[3–18] showed the highest hydrolyzing and the lowest cyclization activities as compared with those of wild-type CGTase I-5.

The mutant had three mutations, M234T, F259I and V591A. The effect of mutation at F259 on increased hydrolyzing activity of CGTase has been reported previously (Fujiiwara et al., 1992b; Nakamura et al., 1994; van der Veen et al., 2001). However, those at M234 and V591 are reported for the first time in this study regarding the activity. M234 and V591 are conserved among various CGTases, whereas V591 is not (Figure 2). None of these residues is conserved among other enzymes in glycoside hydrolase family 13. The introduction of V591A into wild-type CGTase I-5 increased the hydrolyzing activity by 2.4-fold and decreased the cyclization activity only slightly in CGTase[1–7]. The additional introduction of M234T in CGTase[2–28] increased the hydrolyzing activity a further 3-fold without a significant decrease in cyclization activity. However, the introduction of F259I in the final round of PCR further enhanced the hydrolyzing activity by more than another 2-fold and led to a 10-fold reduction in cyclization activity. Mutations at other sites, such as G35S, D39N, A101G, L210V, L551P, R553A and V639I, appeared to be neutral. Sequence alignment of different CGTases indicated that M234 and F259 are located in the vicinity of catalytic sites of the enzyme (Figure 2). M234 is located adjacent to the sugar-binding subsite +1, while F259 constitutes subsite +2 and is known to have critical role in cyclization activity (Uitdehaag et al., 1999; van der Veen et al., 2001). Based on the predicted tertiary structure, T234 is not likely to be located within a distance that could affect substrate binding, but it could cause a change in the loop in which A230, K232 and H233, the residues interacting with the +1 and +2 subsites, are located (Figure 3). According to a recent mutagenesis study of B.circulans 251 CGTase, the residue A230 at subsite +1 plays an important role in the cyclization activity of CGTase (Leemhuis et al., 2003). The authors proposed that the A230V mutation would negatively affect cyclization activity by steric hindrance at subsite +1. Hence the M234T mutation from a bulky amino acid (Met) to a smaller one (Thr) might lead to a change in the position of the peptide backbone near the mutation and could thus alter the torsional angles of A230, K232 and H233. Conclusively, these results suggest that the mutations near the subsite +1 (M234T) and at subsite +2 (F259I) alter enzyme activities in a concerted manner, promoting hydrolysis of substrate while retarding cyclization.

Table I. Summary of the genetic and enzymatic characteristics of the CGTase mutants

<table>
<thead>
<tr>
<th>Mutations</th>
<th>CGTase I-5 (wild-type)</th>
<th>1st round</th>
<th>2nd round</th>
<th>3rd round</th>
</tr>
</thead>
<tbody>
<tr>
<td>G35S</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D39N</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>A101G</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L210V</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M234T</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F259I</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L551P</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R553A</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>V591A</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V639I</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F(%)</td>
<td>100</td>
<td>239</td>
<td>313</td>
<td>785</td>
</tr>
<tr>
<td>C(%)</td>
<td>100</td>
<td>90</td>
<td>81</td>
<td>69</td>
</tr>
<tr>
<td>H/C</td>
<td>1</td>
<td>2.7</td>
<td>3.9</td>
<td>11.4</td>
</tr>
</tbody>
</table>

a: X: mutation site.
b: Relative hydrolyzing activity.
c: Relative cyclization activity.
d: Ratio of hydrolyzing activity to cyclization activity.
The mutant CGTase[3–18] displayed 8% of the cyclization activity and 1500% of the hydrolyzing activity of the wild-type enzyme. The ratio of hydrolyzing activity to cyclization activity in CGTase[3–18] was 197-fold that of wild-type CGTase I-5 (Table II). The $k_m$ values for the two enzymes were not significantly different from each other. Taking the average molecular weight of the substrate as 1$\times$10$^5$ g/mol, $k_m$ for the hydrolyzing activity of wild-type and the mutant was 2.1 and 1.5 $\mu M$, respectively. Using the substrate, $K_m$ for the cyclization activity of wild-type and the mutant was 6.8 and 4.2 $\mu M$, respectively. In the meantime, the $k_{cat}$ for the hydrolyzing activity of CGTase[3–18] increased significantly and the $k_{cat}$ for cyclization activity decreased dramatically in comparison with those of wild-type CGTase (Table III). The effect of each mutation accumulated in CGTase[3–18] was analyzed by using site-directed mutagenesis to construct a mutant enzyme with each mutation (Table II). The hydrolyzing activities of the M234T and F259I mutants were increased by more than 7-fold, and F259I also showed a dramatic decrease in cyclization activity. On the other hand, the effect of V591A on both activities was relatively mild as compared with the effects of the other two mutations. These results suggest that the mutations near subsite +1 (M234T) and at subsite +2 (F259I) also showed a dramatic decrease in cyclization activity.

Effect of the mutant enzyme on CD production and volume of bread loaf

The production of CDs in a bread loaf is undesirable when CGTase is added to the bread mix. HPLC analysis of the bread loaves revealed that α-, β- and γ-CD and G3 were produced in the bread loaf treated with wild-type CGTase I-5 (Figure 4B), whereas no CD was detected in the bread loaf treated with CGTase[3–18] (Figure 4C). CGTase I-5 produced 2.13 mg of β-CD per 1 g of bread crumbs on its addition to bread mix. The fact that CD was not detected in the bread loaf treated with CGTase[3–18] despite its residual cyclization activity (Table II) might have been due to the reaction conditions and structural differences between soluble starch and the starch in the bread mix. Maltose was produced in a larger amount and G3 in a smaller amount in the bread loaf treated with CGTase[3–18] (Figure 4C), as compared with levels in the loaf treated with wild-type CGTase I-5 (Figure 4B). Maltooligosaccharides larger than maltotriose were not detected in the bread loaf treated with CGTase[3–18], indicating very limited disproportionation activity by the mutant enzyme. The bread loaf treated with CGTase ET1 and CGTase ET1-F255I (corresponding to F259 in CGTase I-5) contained significant amounts of maltooligosaccharides with DP of G4–G5 (Lee et al., 2002).

Generally, the volume of a bread loaf is considered to be closely related to the retardation of bread staling. Yeast metabolism is enhanced in the presence of starch hydrolysates produced by the action of added enzymes and consequently more CO$_2$ is produced as the yeast grows, increasing the volume of the bread loaf. Mutsaers and van Eijk (1995) reported that the volume of a bread loaf was increased owing to the production of CD on the addition of CGTase. In this study, wild-type CGTase increased the volume of the bread loaf by 18% when it was added to the bread mix (Figure 5). However, the volume of the bread loaf was increased by 30% when Novamyl or CGTase[3–18] was added to the bread mix. Based on the results obtained in this study, CDs did not seem to play a critical role in increasing the volume of the bread loaf.

Analysis of bread retrogradation

Generally, the first endothermic peak of DSC at ~40–80°C is considered to be the staling endotherm that results from the melting of crystalline amylpectin when starch solution or starchy food is heated (Russell, 1983; Kweon et al., 1994). The degree of bread staling during storage can be monitored using DSC as the increase of the first endothermic peak area, because amylopectin is the main component responsible for retrogradation of starch (Kweon et al., 1994).
The addition of an antistaling enzyme, Novamyl, CGTase I-5 or CGTase[3–18], significantly retarded retrogradation of the bread loaf (Figure 6). Throughout the storage period of 7 days, the retrogradation rates of the experimental loaves were much lower than that of the control loaf to which no enzyme was added. Little difference was found among the retrogradation rates of the bread loaves supplemented with the three enzymes, although the lowest retrogradation rates were seen in the loaf treated with the mutant enzyme CGTase[3–18].

Table II. Effect of each mutation accumulated in CGTase[3–18]

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Optimum temperature (°C)</th>
<th>Hydrolyzing activity (U/mg)</th>
<th>Cyclization activity (U/mg)</th>
<th>H/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGTase I-5 (wild-type)</td>
<td>60</td>
<td>4.1 (100%)</td>
<td>260.0 (100%)</td>
<td>1.0</td>
</tr>
<tr>
<td>CGTase [M234T]</td>
<td>60</td>
<td>30.8 (752%)</td>
<td>210.2 (81%)</td>
<td>9.3</td>
</tr>
<tr>
<td>CGTase [F259I]</td>
<td>50</td>
<td>29.2 (730%)</td>
<td>28.4 (11%)</td>
<td>66.4</td>
</tr>
<tr>
<td>CGTase [V591A]</td>
<td>60</td>
<td>9.8 (239%)</td>
<td>234.1 (90%)</td>
<td>2.7</td>
</tr>
<tr>
<td>CGTase[3–18]</td>
<td>50</td>
<td>64.8 (1580%)</td>
<td>20.1 (8%)</td>
<td>197.5</td>
</tr>
</tbody>
</table>

*Ratio of hydrolyzing activity to cyclization activity.

Table III. Kinetic parameters of the hydrolyzing and cyclization reaction

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_m$ (mg/ml)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (mg/ml)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGTase I-5 (wild-type)</td>
<td>0.21 ± 0.02</td>
<td>5.7 ± 0.3</td>
<td>0.68 ± 0.02</td>
<td>290.4 ± 8.7</td>
</tr>
<tr>
<td>CGTase[3–18]</td>
<td>0.15 ± 0.01</td>
<td>74.6 ± 4.8</td>
<td>0.42 ± 0.02</td>
<td>21.0 ± 1.4</td>
</tr>
</tbody>
</table>

The addition of an antistaling enzyme, Novamyl, CGTase I-5 or CGTase[3–18], significantly retarded retrogradation of the bread loaf (Figure 6). Throughout the storage period of 7 days, the retrogradation rates of the experimental loaves were much lower than that of the control loaf to which no enzyme was added. Little difference was found among the retrogradation rates of the bread loaves supplemented with the three enzymes, although the lowest retrogradation rates were seen in the loaf treated with the mutant enzyme CGTase[3–18].
Therefore, CGTase[3–18] is probably as effective an antistaling agent as Novamyl. The production of maltoligosaccharides and the significant reduction in the molecular weights of amylose and amylpectin are likely to be the main causes of the retarded retrogradation in the bread loaf treated with CGTase. However, no significant increase in maltoligosaccharides was observed in the bread loaves treated with CGTase I-5 or CGTase[3–18], indicating that factors other than the molecular weight of amylpectin might be related to the retarded retrogradation of the bread loaf.

In conclusion, three mutations introduced into CGTase 1–5, M234T, F259I, and V591A, changed the catalytic characteristics of the enzyme in a concerted manner. F259 was already known to play an important role in the cyclization activity of CGTase, whereas M234 and V591 were reported for the first time in this study to be involved in the hydrolyzing and cyclization activities of the enzyme. The significant decrease in the cyclization activity and dramatic increase in the hydrolyzing activity in the mutant indicated the efficiency of error-prone PCR in generating enzymes with desired mutations that could not be predicted based on tertiary structures of enzymes. The mutant enzyme could be used as an antistaling enzyme since it reduced the retrogradation rate of bread as effectively as did the commercial antistaling enzyme without production of CD in bread when added to the bread mix.

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References

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