DOI:10.1021/jf902312u

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

# Mutations of Lysine 47 in Cyclodextrin Glycosyltransferase from *Paenibacillus macerans* Enhance β-Cyclodextrin Specificity

Zhao-Feng Li,[†,#] Jia-Yu Zhang,[†,§] Qi Sun,[†,§] Miao Wang,[†,#] Zheng-Biao Gu,[†,#] Guo-Cheng Du,[†,§] Jing Wu,*[,†,§] and Jian Chen*[,†,§]

[†] State Key Laboratory of Food Science and Technology and [§] School of Biotechnology and Key Laboratory of Industrial Biotechnology, Ministry of Education and [#] School of Food Science and Technology, Jiangnan University, 1800 Lihu Avenue, Wuxi 214122, People's Republic of China

The nature of amino acid residue 47 shows a clear discrimination between the different groups of cyclodextrin glycosyltransferase (CGTase). The effects of amino acid side chain at position 47 on cyclodextrin product specificity were investigated by replacing Lys47 in the CGTase from *Paenibacillus macerans* strain JFB05-01 with arginine, histidine, threonine, serine, or leucine. All of the mutations reduced α-cyclodextrin-forming activity, whereas significant increases in β-cyclodextrin-forming activity were achieved. Especially, the mutations of Lys47 into threonine, serine, or leucine converted *P. macerans* CGTase from α-type to β/α-type. As a result, all of the mutants displayed a shift in product specificity toward the production of β-cyclodextrin. Thus, they were more suitable for the industrial production of β-cyclodextrin than the wild-type enzyme. The enhancement of β-cyclodextrin specificity might be due to weakening or removal of hydrogen-bonding interactions between the side chain of residue 47 and the bent intermediate specific for α-cyclodextrin formation.

**KEYWORDS: Cyclodextrin glycosyltransferase; cyclodextrin; product specificity; *Paenibacillus macerans*; residue 47; mutation**

## INTRODUCTION

Cyclodextrins are cyclic, nonreducing oligo-glucopyranose molecules linked via α(1,4)-glycosidic bonds mainly consisting of six, seven, or eight glucose residues (α-, β-, or γ-cyclodextrin, respectively) (*1*). They can form inclusion complexes with various hydrophobic guest molecules (*2*). As a consequence of complex formation, the properties of the included molecules are altered (*3*,*4*), which leads to many potential applications of cyclodextrins in the industries related to food, pharmaceuticals, chemicals, agriculture, etc. (*5*−*7*). Cyclodextrins are commonly produced by the enzymatic conversion of starch via an intramolecular transglycosylation reaction catalyzed by cyclodextrin glycosyltransferase (EC 2.4.1.19, CGTase). In addition to the cyclization reaction, CGTase also catalyzes three other reactions: coupling, disproportionation, and starch hydrolysis reactions (*8*).

All known wild-type CGTases produce a mixture of α-, β-, and γ-cyclodextrins (*9*, *10*). According to the main cyclodextrin product during the initial stage of starch conversion, the CGTases have been further classified into three main groups: α-, β-, and γ-CGTases (*11*, *12*). The limited cyclodextrin product specificity of CGTase is disadvantageous for cyclodextrin production because the isolation of one particular type of cyclodextrin from the reaction mixture requires a series of additional steps, including selective crystallization of β-cyclodextrin (which is relatively poorly water-soluble) and selective complexation of α- or γ-cyclodextrins with organic solvents (*13*, *14*). The additional steps will increase significantly the costs of cyclodextrin production. Moreover, the use of organic solvents also limits cyclodextrin applications involving human consumption (*14*, *15*). Therefore, the availability of CGTase capable of producing an increased ratio of one particular type of cyclodextrin is highly desired. This situation has strongly stimulated the constructions of mutant CGTases with improved cyclodextrin product specificity.

In the past, a large number of site-directed mutations affecting cyclodextrin product specificity of CGTase have been made (*11*, *16*−*18*). Most of these mutations were based upon amino acid residues located in the active center cleft, which contains at least nine sugar-binding subsites with the catalytic site between subsites +1 and −1 (*19*). Recently, some papers have shown that many mutations at subsite −3 in the CGTases from different sources could change cyclodextrin product specificity (*13*, *16*, *20*−*22*), suggesting that subsite −3 was a key site for cyclodextrin product specificity of CGTase.

The enzyme used in our studies, CGTase from *Paenibacillus macerans*, produces mainly α-cyclodextrin during the initial stage of starch conversion and thus is identified as α-CGTase (*22*). At present, it is the most commonly used enzyme in the commercial production of α-cyclodextrin (*13*). In fact, after prolonged incubation of *P. macerans* CGTase with soluble starch under conditions resembling the industrial production nonsolvent process, the proportion of β-cyclodextrin in the total cyclodextrin products is even higher than that of α-cyclodextrin (*22*), so this

*Corresponding authors [telephone +86-510-85327802; fax +86-510-85327802; e-mail jingwu@jiangnan.edu.cn (J.W.) or jchen@jiangnan.edu.cn (J.C.)].

 Published on Web 08/28/2009 

**Table 1.** Oligonucleotide Primers Used for Site-Directed Mutagenesis

| primer | sequence[a] (5′ to 3′ direction) |
|---|---|
| K47R-For | CGATCCAATTTGCGGCTCTATTTCGG |
| K47R-Rev | CCGAAATAGAGCCGCAAATTGGATCG |
| K47H-For | CGATCCAATTTGCACCTCTATTTCGG |
| K47H-Rev | CCGAAATAGAGGTGCAAATTGGATCG |
| K47T-For | CGATCCAATTTGACGCTCTATTTCGG |
| K47T-Rev | CCGAAATAGAGCGTCAAATTGGATCG |
| K47S-For | CGATCCAATTTGTCACTCTATTTCGG |
| K47S-Rev | CCGAAATAGAGTGACAAATTGGATCG |
| K47L -For | CGATCCAATTTGCTCCTCTATTTCGG |
| K47L -Rev | CCGAAATAGAGGAGCAAATTGGATCG |

[a] Underlined sequences denote the coding sequences of the mutated amino acid.

CGTase is also frequently used in the commercial production of β-cyclodextrin. Thus, mutants of this CGTase capable of producing an increased ratio of β-cyclodextrin are of high industrial interest for the production of β-cyclodextrin.

It is known that residue 47 (*P. macerans* CGTase numbering) is one of the main residues located near subsite −3 (*16*, *23*). A previous study has shown that the mutation of Arg47 in *Bacillus circulans* strain 251 CGTase into a Leu or Gln resulted in a generally decreased β-cyclodextrin-forming activity (*16*), suggesting that residue 47 had an important role in the cyclization reaction. To further investigate the effects of amino acid side chain at position 47 on different cyclodextrin-forming activities and cyclodextrin product specificity of CGTase and eventually obtain the mutants capable of producing an increased ratio of β-cyclodextrin, in the present study, we chose to replace Lys47 in the CGTase from *P. macerans* strain JFB05-01 with arginine, histidine, threonine, serine, or leucine. The relative mechanisms were also analyzed.

## MATERIALS AND METHODS

**Bacterial Strains and Plasmids.** *Escherichia coli* JM109 was used for recombinant DNA manipulations. The (mutant) CGTases were produced with *E. coli* BL21(DE3) harboring (mutant) plasmid *cgt*/pET-20b(+). Plasmid *cgt*/pET-20b(+), in which the gene coding the mature wild-type CGTase from *P. macerans* strain JFB05-01 (CCTCC M203062) was placed downstream of a DNA sequence coding pelB signal peptide and fused in frame to six histidine codons (*24*), was used for site-directed mutagenesis, sequencing, and expression of the (mutant) CGTase proteins.

**DNA Manipulation and Sequencing.** EX Taq HS DNA polymerase, restriction endonucleases, and PCR reagents were purchased from TaKaRa Shuzo (Otsu, Japan). DNA manipulations and calcium chloride transformation of *E. coli* strains were performed as described (*25*). DNA sequences were determined by cycle sequencing with an ABI PRISM BigDye primer cycle sequencing kit with AmpliTaq DNA polymerase (Perkin-Elmer, Foster City, CA). Multiple sequence alignment was performed with the CLUSTAL W program (*26*).

**Site-Directed Mutagenesis.** Site-directed mutagenesis was performed by a one-step PCR method using plasmid *cgt*/pET-20b(+) as template and a pair of complementary primers. The sequences of the mutagenic primers are shown in **Table 1**. PCR products were treated with *Dpn*I and used to transform *E. coli* JM109. The resulting (mutant) plasmids from these clones were transformed into *E. coli* BL21(DE3). DNA sequencing was performed to verify the presence of each of these mutations.

**Production of the (Mutant) CGTases.** A single colony of *E. coli* BL21(DE3) cells harboring (mutant) plasmid *cgt*/pET-20b(+) was inoculated into 10 mL of Luria−Bertani medium in the presence of 100 μg/mL ampicillin and grown at 37 °C overnight. One milliliter of overnight culture was then diluted into 100 mL of terrific broth medium in the presence of 100 μg/mL ampicillin and incubated on a rotary shaker (200 rpm) at 30 °C. The expression of (mutant) CGTases was induced with 0.01 mM isopropyl β-D-1-thiogalactopyranoside added at an optical density at 600 nm of approximately 0.6. Protein induction was performed at 25 °C and continued for 90 h.

| | Bacterial source | Partial sequence 45-50 | Accession No. |
|---|---|---|---|
| α-CGTase | *Klebsiella pneumoniae* M5a1 | NLKKYT | P08704 |
| | *P. macerans* JFB05-01 | NLKLYF | This study |
| | *P. macerans* IFO3490 | NLKLYF | AAC04359 |
| | *Bacillus macerans* | NLKLYF | P31835 |
| | *Thermococcus sp.* B1001 | NWKLYW | AB025721 |
| α/β-CGTase | *T. thermosulfurigenes* EM1 | SLKKYF | AAB00845 |
| | *Thermoanaerobacter sp.* ATCC53.627 | SLKKYF | —— |
| | *B. licheniformis* | NLKLYC | CAA41771 |
| β-CGTase | *B. circulans* 8 | NLKLYC | P30920 |
| | *Bacillus sp.* 6.6.3 | NLKLYC | CAA46901 |
| | *B. circulans* E192 | NLKLYC | —— |
| | Alkalophilic *B. sp.* 38-2 | NLRLYC | P09121 |
| | *Bacillus sp.* B1018 | NLRLYC | P17692 |
| | Alkalophilic *B. sp.* 1011 | NLRLYC | P05618 |
| | *B. circulans* 251 | NLRLYC | CAA55023 |
| β/γ-CGTase | *Bacillus sp.* KC201 | DLHKYC | BAA02380 |
| | Alkalophilic *B. sp.* N 1.1 | DLHKYC | P31746 |
| | *B. ohbensis* C-1400 | DLHKYC | BAA14289 |
| | *Brevibacillus brevis* CD162 | DLHKYC | AAB65420 |
| γ-CGTase | *B. firmus/lentus* 290-3 | DLTKYC | CAA01436 |
| | *B. larkia* 7364 | DLTKYC | BAB91217 |
| | *Bacillus sp.* G-825-6 | DLTKYC | BAE87038 |

**Figure 1.** Multiple sequence alignment of the region around amino acid residue 47 in the CGTases from different sources. The position of residue 47 is shown in bold. Two cyclodextrins are indicated in those cases where both cyclodextrins were formed in comparable amounts, but with (slight) preference for the first one mentioned.

**Purification of the (Mutant) CGTases.** Culture supernatants were obtained by centrifugation. The (mutant) CGTases in the culture supernatant were further purified by affinity chromatography using a Ni-NTA agarose column (Qiagen, Chatsworth, CA). Purity and molecular weight were determined using sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE, 10% gel). The purified enzymes were divided into aliquots and stored at −80 °C. Protein concentration was determined according to the Bradford assay (*27*) using bovine serum albumin (Sigma-Aldrich, Milwaukee, WI) as a standard.

**Assays of CGTase.** All enzyme assays were performed by incubating 0.1 mL of appropriately diluted enzyme with 0.9 mL of 3% (w/v) soluble starch solution in 50 mM phosphate buffer (pH 6.0) at 40 °C for 10 min. α-, β-, and γ-cyclodextrin-forming activities were determined according to the methyl orange (*22*, *28*), phenolphthalein (*29*), and bromocresol green (*30*) methods, respectively. One unit of each activity was defined as the amount of enzyme that was able to produce 1 μmol of the corresponding cyclodextrin per minute.

**Thermostabilities of the (Mutant) CGTases.** The thermostabilities of the (mutant) CGTases were determined by incubating the purified enzymes in 50 mM phosphate buffer (pH 6.0) at 40 °C. At various times, aliquots of the enzyme were removed and assayed for residual α-cyclization activity.

**HPLC Product Analysis.** The formation of cyclodextrins under conditions resembling industrial production process was measured by incubation of 0.2 unit/mL (mutant) CGTases (total cyclization activity) with 5% (wet basis, w/v) soluble starch solution in 50 mM phosphate buffer (pH 6.0) at 40 °C for 40 h. At regular time intervals, samples were taken and boiled for 10 min to terminate the reaction. To eliminate contaminating oligosaccharides, the glucoamylase was added to the boiled sample at a final concentration of 1 unit/mL, and then the mixture was incubated at 30 °C for 2 h, followed by boiling for 10 min. The concentrations of α-, β-, and γ-cyclodextrins in the final sample were determined by HPLC on a Lichrosorb NH2 column (Merck, Darmstadt, Germany) eluted with acetonitrile/water (65:45) at a flow rate of 1 mL/min. Products were detected by a Waters 410 refractive index detector (Waters Corp., Milford, MA).

**Structure Modeling of the (Mutant) CGTases.** The theoretical structures of the (mutant) CGTases were obtained by homology modeling from the SWISS-MODEL protein-modeling server (http://www.expasy.ch/swissmod/SWISS-MODEL.html) (*31*). The proposed complex structures of

**Table 2.** Cyclodextrin-Forming Activities and Thermstabilities (at 40 °C) of the Wild-Type and Mutant CGTases from *P. macerans* Strain JFB05-01[a]

| enzyme | α-cyclodextrin-forming activity (units/mg) | β-cyclodextrin-forming activity (units/mg) | γ-cyclodextrin-forming activity (units/mg) | total (units/mg) | half-life $t_{1/2}$ (h) |
|---|---|---|---|---|---|
| wild-type | 190.4 (85.1) | 32.2 (14.4) | 1.1 (0.5) | 223.7 | 8.0 |
| K47R | 165.5 (77.3) | 46.6 (21.7) | 2.0 (0.9) | 214.1 | 7.2 |
| K47H | 125.8 (62.9) | 71.9 (35.9) | 2.4 (1.2) | 200.1 | 6.5 |
| K47T | 88.1 (47.4) | 95.5 (51.4) | 2.2 (1.2) | 185.8 | 5.9 |
| K47S | 84.5 (46.4) | 95.7 (52.5) | 2.0 (1.1) | 182.2 | 6.2 |
| K47L | 80.1 (45.9) | 92.5 (53.1) | 1.8 (1.0) | 174.4 | 4.8 |

[a] Each value represents the mean of three independent measurements, and the deviation from the mean was below 5%. Numbers in parentheses indicate the ratio in specific activities for the formation of the different cyclodextrins.

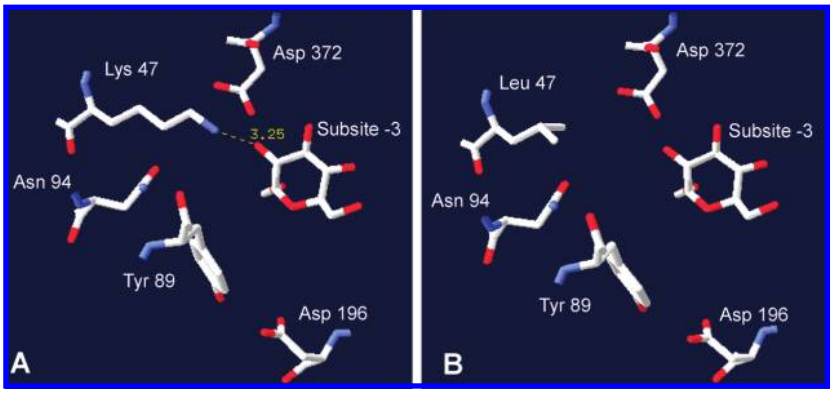


**Figure 2.** Different conformations of amino acid side chain at position 47 in the structural models of the wild-type CGTase (**A**) and mutant K47L (**B**) complexed with a maltohexaose inhibitor in the active site.

the wild-type and mutant CGTases with a maltohexaose inhibitor were modeled by means of superpositioning of the above theoretical structure and the complex structure of *Thermoanaerobacterium thermosulfurigenes* strain EM1 CGTase with a maltohexaose inhibitor in the active site (PDB accession code 1A47) (*13*), followed by least-squares fitting of the Cα atoms.

## RESULTS AND DISCUSSION

**Residue 47 Is Functionally Conserved in CGTases.** Sequence comparisons of CGTases from different sources showed that the nature of residue 47 has a clear discrimination between the different groups of CGTase (**Figure 1**), suggesting that the identity of residue 47 may affect cyclodextrin product specificity of CGTase. In the α- and α/β-CGTases it is a lysine. In the β-CGTases it is either a lysine or an arginine. In the β/γ-CGTases producing virtually no α-cyclodextrin, it is a histidine. Finally, in the γ-CGTase, it is a threonine. It seemed that the CGTase with a relatively short side chain of residue 47 had a preference for the production of larger cyclodextrins.

To investigate the effect of amino acid side chain at position 47 on cyclodextrin product specificity, in the present study, Lys47 near subsite −3 in the α-CGTase from *P. macerans* strain JFB05-01 was replaced by arginine partly conserved in the β-CGTase, histidine conserved in the β/γ-CGTase, and threonine conserved in the γ-CGTase, respectively. In addition, a serine, in which the side chain is shorter than that of threonine and which has intact hydrogen-bonding capability, and a leucine, in which the side chain is longer than that of threonine but which has no hydrogen-bonding capability, were also chosen to replace Lys47.

**Lys47 Is Important for the α-Cyclization Reaction.** The mutants were successfully constructed by site-directed mutagenesis via one-step PCR; all of the mutations were verified by DNA sequencing. The wild-type and mutant CGTases were expressed in *E. coli* BL21 (DE3). There was no significant difference in the expression levels between the recombinant wild-type and the mutant CGTases, as 40−50 mg of the CGTase proteins/L was produced in the flask cultures. Purified (mutant) CGTase proteins were obtained by one-step nickel affinity chromatography on Ni-NTA resin. SDS-PAGE analysis showed that all proteins were purified to apparent homogeneity and displayed an apparent molecular mass of approximately 76 kDa.

The cyclization activities of wild-type and mutant CGTases are shown in **Table 2**. The wild-type CGTase had a relatively high α-cyclodextrin-forming activity. Compared to the wild-type enzyme, mutations K47R and K47H resulted in 13 and 34% decreases in α-cyclodextrin-forming activity, respectively, whereas mutants K47T, K47S, and K47L showed >2-fold decreases in α-cyclodextrin-forming activity. The decreased α-cyclization activities of all the mutants indicated that Lys47 was important for the α-cyclization reaction.

Previously, some crystal soaking experiments, in which CGTase crystals were first soaked in a solution containing the maltononaose inhibitor and subsequently in a solution containing the maltohexaose inhibitor, had revealed that the (mutant) CGTase with a relatively high α-cyclodextrin-forming activity had a preference for the binding of a maltohexaose inhibitor over a maltononaose inhibitor in the active site (*13*, *19*, *20*). In addition, at subsite −3 the maltohexaose binding mode was radically different from the maltononaose binding mode, the former being more bent toward the center (Tyr195) of the active site (*13*, *22*). Thus, the bent conformation of the ligand should be correlated with the formation of α-cyclodextrin. It might represent a (partly) specific intermediate in the α-cyclization reaction (*13*, *20*). Furthermore, X-ray structural analysis of the CGTase from *T. thermosulfurigenes* strain EM1 complexed with a maltohexaose inhibitor in the active site revealed that the bent conformation was stabilized by Lys47 through hydrogen-bonding interactions with the O2 atom of the sugar at subsite −3 (*13*). Therefore, hydrogen-bonding interactions might also exist between Lys47 in the wild-type CGTase from *P. macerans* and the bent intermediate specific for α-cyclodextrin formation (**Figure 2A**). However, it was noted that hydrogen-bonding

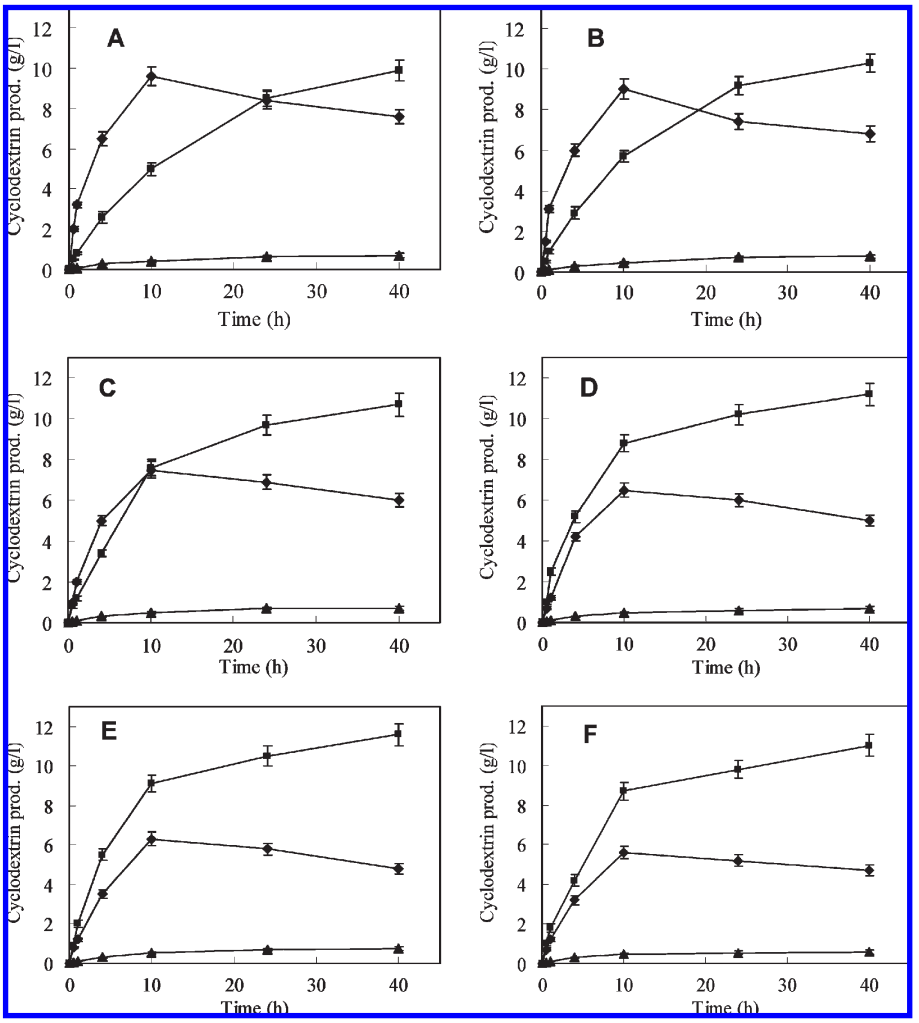


**Figure 3.** Cyclodextrins formed during incubation of a (mutant) CGTase from *P. macerans* strain JFB05-01 (0.2 unit/mL total cyclization activity) with 5% (wet basis, w/v) soluble starch solution at pH 6.0 and 40 °C for 40 h: **A**, wild-type CGTase; **B**, mutant K47R; **C**, mutant K47H; **D**, mutant K47T; **E**, mutant K47S; F, mutant K47L; ◆, α-cyclodextrin; ■, β-cyclodextrin; ▲, γ-cyclodextrin. Each value represents the mean of three independent measurements.

interactions were not found between Arg47 in the β-CGTases and the bent or straight intermediate, although Arg47 could hydrogen bond to cyclic compounds (such as cyclodextrin) (*16*).

In the present study, due to the removal of all possible hydrogen-bonding interactions with the sugar at subsite −3, the K47L mutation might dramatically affect the stabilization of the bent conformation (**Figure 2B**). Moreover, the removal of the hydrophilicity might also affect neighboring amino acids. Thus, among all of the mutants, the K47L mutant showed the highest decrease in α-cyclodextrin-forming activity. Mutations of Lys47 into threonine or serine, with intact hydrogen-bonding capability but a much shorter side chain than the original lysine, might also cause the almost complete loss of the interactions between the side chain of residue 47 and the bent intermediate, thus resulting in significant decreases in α-cyclodextrin-forming activity, which were comparable to the K47L mutation. In addition, mutations of Lys47 into arginine or histidine caused moderate decreases in α-cyclodextrin-forming activity when compared to the wild-type enzyme and mutant K47L. This suggested that the interactions for Lys47 with the bent intermediate were at least partially retained in both mutants.

**Mutations of Lys47 Increased β-Cyclization Activity.** The wild-type CGTase had a relatively low β-cyclodextrin-forming activity (**Table 2**). Significant increases in β-cyclodextrin-forming activity were, however, achieved by mutations of Lys47 (**Table 2**). Compared to the wild-type enzyme, mutations K47R and K47H resulted in 1.5- and 2.2-fold increases in β-cyclodextrin-forming activity, respectively, whereas mutants K47T, K47S, and K47L showed about 3-fold increases in β-cyclodextrin-forming activity, which was even higher than α-cyclodextrin-forming activity. Probably due to the destabilization of the bent conformation, the intermediates in a straight conformation, which might be (partly) specific for the formation of β-cyclodextrin (*19*, *22*, *32*), were more frequently formed in the cyclization reaction of these mutants. Noteworthily, in the *B. circulans* strain 251 CGTase, the mutations of Arg47, even into Leu, displayed contrary effects on β-cyclodextrin-forming activity (*16*), suggesting that the responses of CGTases from different sources to the mutations of residue 47 were significantly different.

The wild-type CGTase showed little detectable γ-cyclodextrin-forming activity, whereas γ-cyclodextrin-forming activity was not affected significantly by all of the mutations of Lys47 (**Table 2**). A possible explanation was that these mutations did not produce more space at the end of the substrate binding cleft to accommodate additional sugar residues (*33*).

However, for all of the mutants, the increase in β-cyclodextrin-forming activity did not completely compensate for the decrease in α-cyclodextrin-forming activity. As a result, the mutations of Lys47 resulted in 4−22% decreases in total cyclization activity. Furthermore, the mutant with a lower α-cyclodextrin-forming activity corresponded to a lower total cyclization activity.

**Table 3.** Starch Conversion of the Wild-Type and Mutant CGTases from *P. macerans* Strain JFB05-01[a]

| CGTase | conversion of starch into cyclodextrins (%) | product (g/L) | | |
|---|---|---|---|---|
| | | α | β | γ |
| wild-type | 42.3 | 7.6 (41.8) | 9.9 (54.4) | 0.69 (3.8) |
| K47R | 41.5 | 6.8 (38.1) | 10.3 (57.7) | 0.75 (4.2) |
| K47H | 40.5 | 6.0 (34.4) | 10.7 (61.4) | 0.72 (4.1) |
| K47T | 39.3 | 5.0 (29.6) | 11.2 (66.4) | 0.68 (4.0) |
| K47S | 39.8 | 4.8 (28.0) | 11.6 (67.7) | 0.73 (4.3) |
| K47L | 37.9 | 4.7 (28.0) | 11.0 (67.6) | 0.58 (4.4) |

[a] CGTase proteins (0.2 unit/mL total cyclization activity) were incubated with 5% (wet basis, w/v) soluble starch solution at pH 6.0 and 40 °C for 40 h. Numbers in parentheses indicate the ratio in the different cyclodextrins.

**Mutations of Lys47 Enhanced β-Cyclodextrin Specificity.** The effect of the mutations of Lys47 on cyclodextrin product specificity was first reflected in the change of initial cyclodextrin product ratio. Among all four CGTase-catalyzed reactions, only the effect of cyclization is apparent at the start of the reaction (*16*, *20*). Thus, this ratio is determined by different cyclodextrin-forming activities of CGTase and reflects the preference of the enzyme for the production of the specific cyclodextrins (*20*). In the present study, all of the mutations of Lys47 resulted in increased proportions of β-cyclodextrin-forming activity in total cyclization activity (**Table 2**), thus causing a shift in the preference toward the production of β-cyclodextrin. The shift was most apparent for mutants K47T, K47S, and K47L, which showed a preference for the production of β-cyclodextrin over α-cyclodextrin. It was suggested that these single mutations could convert *P. macerans* CGTase from α-type into β/α-type. However, these results were not consistent with the expectation, obtained from multiple sequence alignment (**Figure 1**), that the mutant with a shorter side chain of residue 47 had a higher preference for the production of larger cyclodextrins, whereas hydrogen-bonding interactions between the side chain of residue 47 and the bent intermediate in the α-cyclization reaction appeared to contribute to the size of cyclodextrin products formed (**Figure 2**).

The mutations of Lys47 also resulted in the change of cyclodextrin product ratio after equilibrium was reached. For the wild-type CGTase, at the initial stage of the reaction, α-cyclodextrin was the main product. At the later stages, the proportion of α-cyclodextrin in the total cyclodextrin products decreased while the proportion of β-cyclodextrin increased (**Figure 3A**). After 40 h of incubation, the wild-type enzyme produced a mixture of α-, β-, and γ-cyclodextrins at a ratio of 41.8:54.4:3.8 (**Table 3**). Compared to the wild-type enzyme, all of the mutants produced more β-cyclodextrin at the expense of α-cyclodextrin, whereas the production of γ-cyclodextrin was not affected significantly (**Figure 3B−F**). Especially for the mutants K47T, K47S, and K47L, after 40 h of incubation, the amounts of β-cyclodextrin in the reaction mixture were about 1.1−1.2-fold of that of the wild-type enzyme, whereas the amounts of α-cyclodextrin decreased to 62−66% of that of the wild-type enzyme (**Table 3**). The proportions of β-cyclodextrin in the total cyclodextrin products reached 66−68% (**Table 3**).

The final cyclodextrin product ratio is a combined result of all four CGTase-catalyzed reactions (*13*, *20*, *34*). With the incubation time prolonged, the degradation of cyclodextrins via the coupling reaction is not negligible. Meanwhile, α-, β-, and γ-cyclodextrin formation rates will gradually decrease due to product inhibition (*22*). Eventually, the formation and degradation of α-, β-, and γ-cyclodextrins may reach equilibrium. For any (mutant) CGTase, the final ratio obtained after 40 h of incubation (**Table 3**) had an obvious difference from the initial ratio expected from its cyclodextrin-forming activities (**Table 2**). Nevertheless, the changes of the final ratio for the mutants could be closely related to their altered cyclodextrin-forming activities; after 40 h of incubation, the mutant with a higher proportion of β-cyclodextrin-forming activity in total cyclization activity (**Table 2**) still had an increased contribution of β-cyclodextrin at the expense of α-cyclodextrin when compared to the wild-type enzyme (**Table 3**).

In addition, although equal amounts of cyclization activity were used in our cases, all of the mutants had lower conversions of starch into cyclodextrins than the wild-type enzyme (**Table 3**). Stability tests revealed that the mutant with a lower conversion was accompanied by a shorter half-life at 40 °C (**Table 2**), so the decreased total cyclodextrin production from starch was probably caused by the decreased stability of the enzyme.

In summary, all of the mutations described in this study resulted in significant increases in β-cyclodextrin-forming activities, with concomitant decreases in α-cyclodextrin-forming activity. Especially, the mutations of Lys47 into threonine, serine, or leucine could convert *P. macerans* CGTase from α-type into β/α-type. As a result, these mutants displayed a shift in product specificity toward the production of β-cyclodextrin. They were more suitable for the industrial production of β-cyclodextrin than the wild-type enzyme. These data provided clear evidence that amino acid side chain at position 47 near subsite −3 had an important effect on the cyclodextrin product specificity of CGTase.

## LITERATURE CITED


(1) Li, Z. F.; Li, B.; Liu, Z. G.; Wang, M.; Gu, Z. B.; Du, G. C.; Wu, J.; Chen, J. Calcium leads to further increase in the glycine-enhanced extracellular secretion of recombinant α-cyclodextrin glycosyltransferase in *Escherichia coli*. *J. Agric. Food Chem.* **2009**, *57*, 6231–6237.

(2) Saenger, W. Cyclodextrin inclusion compounds in research and industry. *Angew. Chem.* **1980**, *19*, 344–362.

(3) Lucas-Abellan, C.; Gabaldon-Hernandez, J. A.; Penalva, J.; Fortea, M. I.; Nunez-Delicado, E. Preparation and characterization of the inclusion complex of chlorpyrifos in cyclodextrins to improve insecticide formulations. *J. Agric. Food Chem.* **2008**, *56*, 8081–8085.

(4) Mourtzinos, I.; Salta, F.; Yannakopoulou, K.; Chiou, A.; Karathanos, V. T. Encapsulation of olive leaf extract in β-cyclodextrin. *J. Agric. Food Chem.* **2007**, *55*, 8088–8094.

(5) Szente, L.; Szejtli, J. Cyclodextrins as food ingredients. *Trends Food Sci. Technol.* **2004**, *15* (3−4), 137–142.

(6) Davis, M. E.; Brewster, M. E. Cyclodextrin-based pharmaceutics: past, present and future. *Nat. Rev. Drug Discov.* **2004**, *3*, 1023–1035.

(7) LeBlanc, B. W.; Boue, S.; De-Grandi Hoffman, G.; Deeby, T.; McCready, H.; Loeffelmann, K. β-Cyclodextrins as carriers of monoterpenes into the hemolymph of the honey bee (*Apis mellifera*) for integrated pest management. *J. Agric. Food Chem.* **2008**, *56*, 8565–8573.

(8) van der Veen, B. A.; van Alebeek, G. J.; Uitdehaag, J. C.; Dijkstra, B. W.; Dijkhuizen, L. The three transglycosylation reactions catalyzed by cyclodextrin glycosyltransferase from *Bacillus circulans* (strain 251) proceed via different kinetic mechanisms. *Eur. J. Biochem.* **2000**, *267*, 658–665.

(9) Li, Z. F.; Wang, M.; Wang, F.; Gu, Z. B.; Du, G. C.; Wu, J.; Chen, J. γ-Cyclodextrin: a review on enzymatic production and applications. *Appl. Microbiol. Biotechnol.* **2007**, *77*, 245–255.

(10) Jeang, C. L.; Lin, D. G.; HsiehI, S. H. Characterization of cyclodextrin glycosyltransferase of the same gene expressed from *Bacillus macerans*, *Bacillus subtilis*, and *Escherichia coli*. *J. Agric. Food Chem.* **2005**, *53*, 6301–6304.
(11) Penninga, D.; Strokopytov, B.; Rozeboom, H. J.; Lawson, C. L.; Dijkstra, B. W.; Bergsma, J.; Dijkhuizen, L. Site-directed mutations in tyrosine 195 of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251 affect activity and product specificity. *Biochemistry* **1995**, *34*, 3368–3376.
(12) Qi, Q.; Zimmermann, W. Cyclodextrin glucanotransferase: from gene to applications. *Appl. Microbiol. Biotechnol.* **2005**, *66*, 475–485.
(13) Wind, R. D.; Uitdehaag, J. C.; Buitelaar, R. M.; Dijkstra, B. W.; Dijkhuizen, L. Engineering of cyclodextrin product specificity and pH optima of the thermostable cyclodextrin glycosyltransferase from *Thermoanaerobacterium thermosulfurigenes* EM1. *J. Biol. Chem.* **1998**, *273*, 5771–5779.
(14) van der Veen, B. A.; Uitdehaag, J. C.; Dijkstra, B. W.; Dijkhuizen, L. Engineering of cyclodextrin glycosyltransferase reaction and product specificity. *Biochim. Biophys. Acta: Protein Struct. Mol. Enzymol.* **2000**, *1543*, 336–360.
(15) Pedersen, S.; Dijkhuizen, L.; Dijkstra, B. W.; Jensen, B. F.; Jorgensen, S. T. A better enzyme for cyclodextrins. *CHEMTECH* **1995**, *25*, 19–25.
(16) van der Veen, B. A.; Uitdehaag, J. C.; Dijkstra, B. W.; Dijkhuizen, L. The role of arginine 47 in the cyclization and coupling reactions of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251 implications for product inhibition and product specificity. *Eur. J. Biochem.* **2000**, *267*, 3432–3441.
(17) Fujiwara, S.; Kakihara, H.; Sakaguchi, K.; Imanaka, T. Analysis of mutations in cyclodextrin glucanotransferase from *Bacillus stearothermophilus* which affect cyclization characteristics and thermostability. *J. Bacteriol.* **1992**, *174* (22), 7478–7481.
(18) Sin, K. A.; Nakamura, A.; Masaki, H.; Matsuura, Y.; Uozumi, T. Replacement of an amino acid residue of cyclodextrin glucanotransferase of *Bacillus ohbensis* doubles the production of γ-cyclodextrin. *J. Biotechnol.* **1994**, *32*, 283–288.
(19) Strokopytov, B.; Knegtel, R. M.; Penninga, D.; Rozeboom, H. J.; Kalk, K. H.; Dijkhuizen, L.; Dijkstra, B. W. Structure of cyclodextrin glycosyltransferase complexed with a maltononaose inhibitor at 2.6 angstrom resolution. Implications for product specificity. *Biochemistry* **1996**, *35*, 4241–4249.
(20) van der Veen, B. A.; Uitdehaag, J. C.; Penninga, D.; van Alebeek, G. J.; Smith, L. M.; Dijkstra, B. W.; Dijkhuizen, L. Rational design of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251 to increase α-cyclodextrin production. *J. Mol. Biol.* **2000**, *296*, 1027–1038.
(21) Kim, Y. H.; Bae, K. H.; Kim, T. J.; Park, K. H.; Lee, H. S.; Byun, S. M. Effect on product specificity of cyclodextrin glycosyltransferase by site-directed mutagenesis. *Biochem. Mol. Biol. Int.* **1997**, *41*, 227–234.
(22) Li, Z. F.; Zhang, J. Y.; Wang, M.; Gu, Z. B.; Du, G. C.; Li, J. K.; Wu, J.; Chen, J. Mutations at subsite −3 in cyclodextrin glycosyltransferase from *Paenibacillus macerans* enhancing α-cyclodextrin specificity. *Appl. Microbiol. Biotechnol.* **2009**, *83*, 483–490.
(23) Uitdehaag, J. C.; Kalk, K. H.; van Der Veen, B. A.; Dijkhuizen, L.; Dijkstra, B. W. The cyclization mechanism of cyclodextrin glycosyltransferase (CGTase) as revealed by a γ-cyclodextrin-CGTase complex at 1.8-A resolution. *J. Biol. Chem.* **1999**, *274*, 34868–34876.
(24) Chen, J.; Wu, J.; Li, Z. F.; Li, B.; Cheng, C. Cloning and expression of the gene encoding α-cyclodextrin glycosyltransferase. CN Patent 200810024162.3, **2008**.
(25) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning, A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989.
(26) Thompson, J. D.; Gibson, T. J.; Higgins, D. G. Multiple sequence alignment using ClustalW and ClustalX. *Curr. Protoc. Bioinform.* **2002**, Chapter 2, Unit 2-3.
(27) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein−dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.
(28) Lejeune, A.; Sakaguchi, K.; Imanaka, T. A spectrophotometric assay for the cyclization activity of cyclomaltohexaose (α-cyclodextrin) glucanotransferase. *Anal. Biochem.* **1989**, *181*, 6–11.
(29) Makela, M.; Korpela, T.; Laakso, S. Colorimetric determination of β-cyclodextrin: two assay modifications based on molecular complexation of phenolphtalein. *J. Biochem. Biophys. Methods* **1987**, *14*, 85–92.
(30) Kato, T.; Horikoshi, K. Colorimetric determination of γ-cyclodextrin. *Anal. Chem.* **1984**, *56*, 1738–1740.
(31) Arnold, K.; Bordoli, L.; Kopp, J.; Schwede, T. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics* **2006**, *22*, 195–201.
(32) Uitdehaag, J. C.; Mosi, R.; Kalk, K. H.; van der Veen, B. A.; Dijkhuizen, L.; Withers, S. G.; Dijkstra, B. W. Catalysis in the α-amylase family-X-ray structures along the reaction pathway of cyclodextrin glycosyltransferase. *Nat. Struct. Biol.* **1999**, *6*, 432–436.
(33) Parsiegla, G.; Schmidt, A. K.; Schulz, G. E. Substrate binding to a cyclodextrin glycosyltransferase and mutations increasing the γ-cyclodextrin production. *Eur. J. Biochem.* **1998**, *255*, 710–717.
(34) Terada, Y.; Yanase, M.; Takata, H.; Takaha, T.; Okada, S. Cyclodextrins are not the major cyclic α-1,4-glucans produced by the initial action of cyclodextrin glucanotransferase on amylose. *J. Biol. Chem.* **1997**, *272*, 15729–15733.

**Received March 18, 2009. Revised manuscript received August 13, 2009. Accepted August 14, 2009. This work was supported financially by the Natural Science Foundation of Jiangsu Province (BK2007019), the National Science Fund for Distinguished Young Scholars (20625619), the National High-tech Research and Development Program of China (863 Program, 2006AA10Z335), the Research Program of State Key Laboratory of Food Science and Technology (SKLF-MB-200802), and the Program of Innovation Team of Jiangnan University (2008CXTD01).**