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Construction of chimeric cyclodextrin glucanotransferases from *Bacillus circulans* A11 and *Paenibacillus macerans* IAM1243 and analysis of their product specificity

Vichien Rimphanitchayakit,[a,*] Takashi Tonozuka[b] and Yoshiyuki Sakano[b]

[a]*Department of Biochemistry, Faculty of Science, Chulalongkorn University, Phyathai Road, Bangkok 10330, Thailand*
[b]*Department of Applied Biological Science, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo 183-8509, Japan*

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Abstract—Three DNA fragments of 7919 base pairs containing genes for β-cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19), an iron III dicitrate transport protein-like protein and a partial coding sequence for putative ferrichrome ABC transporter from *Bacillus circulans* A11 were cloned and sequenced (GenBank Accession AF302787). The DNA sequence contained a CGTase open reading frame of 2139 base pairs, which encoded a polypeptide of 713 amino acid residues. The signal peptide constituted the N-terminal 27 amino acid residues. The amino acid sequence was highly homologous to that of *Bacillus* sp. 1011 with 98.7% identity. The cloned CGTase gene contained its own promoter that directed the expression of the gene in *Escherichia coli* host cells. Chimeric construction against the α-CGTase from *B. macerans* IAM1243 was carried out by means of three created restriction sites, *Xho*I, *Spe*I, and *Mfe*I, introduced by mutagenesis in between domains A/B and C, C and D, and D and E, respectively, and the *Nde*I site within the domains A/B. The various chimeras with different combinations of domains and part of domains A/B were analyzed for their dextrinizing and CD-forming activities. Their activities were of three groups: chimeras with no dextrinizing and cyclization activities, chimeras with only dextrinizing activity, and chimeras with both dextrinizing and cyclization activities. Two chimeras in the latter group showed altered product specificity. The results located the amino acid segment essential for the product specificity at the C-terminal half of domains A/B. Further, the function of domains C and D in positioning domain E in the correct orientation and proximity to domains A/B is implicated.

Keywords: Cyclodextrin glucanotransferase; Cyclodextrin; *Bacillus circulans* A11; Chimeric construction; Product specificity

1. Introduction

Cyclodextrins (CDs) are well known cyclic oligosaccharides, used as carrier molecules in several industries, particularly food, cosmetics, toiletry, and pharmaceutical.[1,2] Three major CDs in the market are α-, β-, and γ-CDs, which, respectively, consist of 6, 7, and 8 glucose units, linked by α-1,4-glycosidic bonds. The cyclic arrangement of the oligosaccharides results in a hydrophilic ring with hydrophobic cavity inside, that is able to form inclusion complexes with several nonpolar chemicals. Those chemicals are thus stabilized and the complexes may give the desired physical and chemical properties for industrial applications.

Cyclodextrins are the products of intramolecular transglycosylation or cyclization reaction of starch chain, catalyzed by an enzyme cyclodextrin glucanotransferase (CGTase). The enzymes are synthesized by many bacterial genera, particularly the Bacilli. All natural CGTases usually produce a mixture of α-, β-, and γ-CDs at different ratios depending on the source of CGTases used. Hence, the names of the CGTases are α-, β-, and γ-CGTases depending on the major product produced. Beside the cyclization reaction, CGTase also

* Corresponding author. Tel.: +66 22185436; fax: +66 22185418; e-mail: kpvr2@yahoo.com

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catalyzes intermolecular transglycosylation reactions, namely, the coupling reaction, the cleavage of the CD ring and joining to a receptor sugar molecule, and disproportionation reaction, the cleavage of straight-chain oligosaccharide and joining to a receptor sugar molecule, and the weak starch hydrolysis reaction.

The three-dimensional structures of five CGTases from *Bacillus circulans* strain 8,[3] *Bacillus* sp. strain 1011,[4] *B. circulans* strain 251,[5] *Thermoanaerobacterium thermosulfurigenes* strain EM1,[6] and a strain of *Bacillus stearothermophilus* (PDB: 1CYG) have been solved. All of which consist of five domains, A, B, C, D, and E, with common domain structure and organization. Domain A comprises the central $(\beta/\alpha)_8$ barrel with the protruding domain B, which together forms the portion of substrate binding and catalytic subsites.[7] Domain E is a starch-binding domain. The functions of domains C and D, however, are not known. The catalytic domain of the CGTase, sometimes referred to as domains A/B, is the important domain for the CD-producing activity as well as the CD product specificity.

Since almost all of the CGTases produce a combination of three major CDs, processes to separate each CD are unavoidable, which are time consuming, cause more expenses, possibly deleterious to the environment, and hazardous to the consumers. Attempts have been made to create CGTases, each with one product specificity, by using the knowledge from the three-dimensional structures of the enzymes and mutagenesis. Changes of amino acid residues at certain positions in the catalytic domain of CGTases to different amino acids have been shown to affect the CD product ratio.[8–12] To date, however, no CGTase with only one product specificity has been successfully engineered.

We report herein the cloning of a β-CGTase from *B. circulans* A11 and, along with the α-CGTase that has different product specificity, the construction of series of chimeric CGTases. The product ratios obtained from the reactions of the chimeras are compared. The amino acid sequence segment located in the C-terminal half of domains A/B is shown to be important for product specificity.

2. Results

2.1. Cloning of cyclodextrin glucanotransferase gene from *B. circulans* A11

In order to clone the cyclodextrin glucanotransferase (CGTase) gene from *B. circulans* A11, a CGTase gene-specific PCR fragment was prepared using the degenerate primers designed from the gene sequences homologous among CGTase genes. The PCR fragment of about 850 base pairs was amplified from the chromosomal DNA from *B. circulans* A11 and cloned into the pGEM®-T vector. The nucleotide sequence was determined in order to verify the identity of the clone. The PCR product was found to be a portion of the CGTase gene from *B. circulans* A11 (underlined sequence in Fig. 2). The PCR fragment was used as a probe in Southern blot hybridization for the isolation of DNA fragments containing CGTase gene and colony hybridization for the screening of CGTase-containing clones (data not shown). Three positive clones, pVR301, 302, and 308, were identified. The DNA inserts were restriction mapped as shown in Figure 1. They were, however, tested inactive in dextrinizing activity suggesting that they did not contain the complete CGTase gene. By examining the restriction map of the CGTase gene-specific PCR fragment, it was predicted that the CGTase gene was in between the pVR302 and 308 inserts. A plasmid pVR328 was then constructed by combining the 1226 bp *Hin*dIII-*Sph*I fragment from pVR302 with the 1835 bp *Sph*I-*Cla*I fragment from pVR308. The pVR328 was active in dextrinization and CD-production. Although the CGTase gene in pVR328 was in the same direction as the *lac* promoter in the pUC119 vector, such promoter did not drive the expression of CGTase since the CGTase activity was observed either in the absence or presence of an inducer, isopropyl thiogalactoside. DNA sequencing revealed that the CGTase gene was indeed cloned along with its own promoter that was active in *Escherichia coli* host cells (Fig. 2).

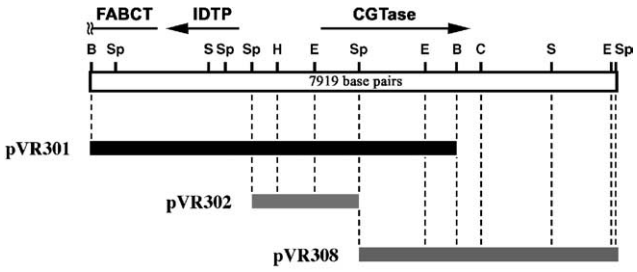

Figure 1. Schematic representation of the DNA inserts from the three hybridization positive clones, pVR301, pVR302, and pVR308. A cyclodextrin glucanotransferase gene, a gene encoding for iron III dicitrate transport protein-like protein (IDTP) and a partial coding sequence for a putative ferrichrome ABC transporter (FABCT) in a total of 7919 base pairs are shown as arrows/incomplete arrow above the restriction map. B = *Bam*HI, C = *Cla*I, E = *Eco*RI, H = *Hin*dIII, S = *Sal*I, and Sp = *Sph*I.

The three positive clones were subjected to subcloning for DNA sequencing. A total of 7919 base pairs were determined (GenBank Accession AF302787). By sequence comparison using Blastx 2.2.10,[13] two complete coding sequences for a CGTase and an iron III dicitrate transport protein-like protein, and a partial coding sequence for a putative ferrichrome ABC transporter were identified (Fig. 1). Part of the DNA sequence containing the CGTase gene from *B. circulans* A11 is shown

```
3371  GAATTCGCTTTCATATAAAATGAACAAGAACACATCACTATACTTACATACAAGCTAAGGGCTATGCATT  3440
3441  CCTTACCTTACCCCGGTATGGAACAACCCCGGTATCTCTATTAGAGACGCCGGGGTTTTTTATGTAGCCG  3510
                  -35                      -10                      SD
3511  AGATGAAGGAGGTGATCCCCAAAGCGACGGACAGGCCTGTTATCCCCAAGCATTGTATACGATGAGGAGG  3580
3581  TATAGTATGAAAAGATTTATGAAACTAACAGCCGTATGGACACTCTGGTTATCCCTCACGCTGGGCCTCT  3650
1           M  K  R  F  M  K  L  T  A  V  W  T  L  W  L  S  L  T  L  G  L  L    22
3651  TGAGCCCGGTCCACGCAGCCCCGGATACCTCGGTATCCAACAAGCAGAATTTCAGCACGGATGTCATATA  3720
23      S  P  V  H  A  A  P  D  T  S  V  S  N  K  Q  N  F  S  T  D  V  I  Y    45
3721  TCAGATCTTCACCGACCGGTTCTCGGACGGCAATCCGGCCAACAATCCGACCGGCGCGGCATTTGACGGA  3790
46     Q  I  F  T  D  R  F  S  D  G  N  P  A  N  N  P  T  G  A  A  F  D  G    68
3791  TCATGTACGAATCTTCGCTTATACTGCGGCGGCGACTGGCAAGGCATCATCAACAAAATCAACGACGGTT  3860
69    S  C  T  N  L  R  L  Y  C  G  G  D  W  Q  G  I  I  N  K  I  N  D  G  Y    92
3861  ATTTGACCGGCATGGGCATTACGGCCATCTGGATTTCACAGCCTGTCGAGAATATCTACAGCGTGATCAA  3930
93      L  T  G  M  G  I  T  A  I  W  I  S  Q  P  V  E  N  I  Y  S  V  I  N   115
3931  CTACTCCGGCGTCCATAATACGGCTTATCACGGCTACTGGGCGCGGGACTTCAAGAAGACCAATCCGGCC  4000
116    Y  S  G  V  H  N  T  A  Y  H  G  Y  W  A  R  D  F  K  K  T  N  P  A   138
4001  TACGGAACGATGCAGGACTTCAAAAACCTGATCGACACCGCGCATGCGCATAACATAAAAGTCATCATCG  4070
139   Y  G  T  M  Q  D  F  K  N  L  I  D  T  A  H  A  H  N  I  K  V  I  I  D   162
4071  ACTTTGCACCGAACCATACATCTCCGGCTTCTTCGGATGATCCTTCCTTTGCAGAGAACGGCCGCTTGTA  4140
163     F  A  P  N  H  T  S  P  A  S  S  D  D  P  S  F  A  E  N  G  R  L  Y   185
4141  CGATAACGGCAACCTGCTCGGCGGATACACCAACGATACCCAAAATCTGTTCCACCATTATGGCGGCACG  4210
186    D  N  G  N  L  L  G  G  Y  T  N  D  T  Q  N  L  F  H  H  Y  G  G  T   208
4211  GATTTCTCCACCATTGAGAACGGCATTTATAAAAACCTGTACGATCTGGCTGACCTGAATCATAACAACA  4280
209   D  F  S  T  I  E  N  G  I  Y  K  N  L  Y  D  L  A  D  L  N  H  N  N  S   232
4281  GCAGCGTCGATGTGTATCTGAAGGATGCCATCAAAATGTGGCTCGACCTCGGGGTTGACGGCATTCGCGT  4350
234     S  V  D  V  Y  L  K  D  A  I  K  M  W  L  D  L  G  V  D  G  I  R  V   255
4351  GGACGCGGTCAAGCATATGCCATTCGGCTGGCAGAAGAGCTTTATGTCCACCATTAACAACTACAAGCCG  4420
256    D  A  V  K  H  M  P  F  G  W  Q  K  S  F  M  S  T  I  N  N  Y  K  P   278
4421  GTCTTCACCTTCGGCGAATGGTTCCTTGGCGTCAATGAGATTAGTCCGGAATACCATCAATTCGCTAACG  4490
279   V  F  T  F  G  E  W  F  L  G  V  N  E  I  S  P  E  Y  H  Q  F  A  N  E   302
4491  AGTCCGGGATGAGCCTGCTCGATTTCCGCTTTGCCCAGAAGGCCCGGCAAGTGTTCAGGGACAACACCGA  4560
303     S  G  M  S  L  L  D  F  R  F  A  Q  K  A  R  Q  V  F  R  D  N  T  D   325
4561  CAATATGTACGGCCTGAAAGCGATGCTGGAGGGCTCTGAAGTAGACTATGCCCAGGTGAATGACCAGGTG  4630
326    N  M  Y  G  L  K  A  M  L  E  G  S  E  V  D  Y  A  Q  V  N  D  Q  V   348
4631  ACCTTCATCGACAATCATGACATGGAGCGTTTCCACACCAGCAATGGCGACAGACGGAAGCTGGAGCAGG  4700
349   T  F  I  D  N  H  D  M  E  R  F  H  T  S  N  G  D  R  R  K  L  E  Q  A   372
4701  CGCTGGCCTTTACCCTGACTTCACGCGGTGTGCCTGCCATCTATTACGGCAGCGAGCAGTATATGTCTGG  4770
373     L  A  F  T  L  T  S  R  G  V  P  A  I  Y  Y  G  S  E  Q  Y  M  S  G   395
4771  CGGGAATGATCCGGACAACCGTGCTCGGATTCCTTCCTTCTCCACGACGACGACCGCATATCAAGTCATC  4840
396    G  N  D  P  D  N  R  A  R  I  P  S  F  S  T  T  T  T  A  Y  Q  V  I   418
4841  CAAAAGCTCGCTCCGCTCCGCAAATCCAACCCGGCCATCGCTTACGGTTCCACACAGGAGCGCTGGATCA  4910
419   Q  K  L  A  P  L  R  K  S  N  P  A  I  A  Y  G  S  T  Q  E  R  W  I  N   442
4911  ACAACGATGTGATCATCTATGAACGCAAATTCGGCAATAACGTGGCCGTTGTTGCCATTAACCGCAATAT  4980
443     N  D  V  I  I  Y  E  R  K  F  G  N  N  V  A  V  V  A  I  N  R  N  M   465
4981  GAACACACCGGCTTCGATTACCGGCCTTGTCACTTCCCTCCCGCAGGGCAGCTATAACGATGTGCTCGGC  5050
466    N  T  P  A  S  I  T  G  L  V  T  S  L  P  Q  G  S  Y  N  D  V  L  G   488
5051  GGAATTCTGAACGGCAATACGCTAACCGTGGGTGCTGGCGGTGCAGCTTCCAACTTTACTTTGGCTCCTG  5120
489   G  I  L  N  G  N  T  L  T  V  G  A  G  G  A  A  S  N  F  T  L  A  P  G   512
5121  GCGGCACTGCTGTATGGCAGTACACAACCGATGCCACAGCTCCGATCATCGGCAATGTCGGCCCGATGAT  5190
513     G  T  A  V  W  Q  Y  T  T  D  A  T  A  P  I  I  G  N  V  G  P  M  M   535
5191  GGCCAAGCCAGGGGTCACGATTACGATTGACGGCCGCGGCTTCGGCTCCGGCAAGGGAACGGTTTACTTC  5260
536    A  K  P  G  V  T  I  T  I  D  G  R  G  F  G  S  G  K  G  T  V  Y  F   558
5261  GGTACAACGGCAGTCACTGGCGCGGACATCGTAGCTTGGGAAGATACACAAATCCAGGTGAAAATCCCTG  5330
559   G  T  T  A  V  T  G  A  D  I  V  A  W  E  D  T  Q  I  Q  V  K  I  P  A   582
5331  CGGTCCCTGGCGGCATCTATGATATCAGAGTTGCCAACGCAGCCGGAGCAGCCAGCAACATCTACGACAA  5400
583     V  P  G  G  I  Y  D  I  R  V  A  N  A  A  G  A  A  S  N  I  Y  D  N   605
5401  TTTCGAGGTGCTGACCGGAGACCAGGTCACCGTTCGGTTCGTAATCAACAATGCCACAACGGCGCTGGGA  5470
606    F  E  V  L  T  G  D  Q  V  T  V  R  F  V  I  N  N  A  T  T  A  L  G   628
5471  CAGAATGTGTTCCTCACGGGCAATGTCAGCGAGCTGGGCAACTGGGATCCGAACAACGCGATCGGCCCGA  5540
629   Q  N  V  F  L  T  G  N  V  S  E  L  G  N  W  D  P  N  N  A  I  G  P  M   652
5541  TGTATAATCAGGTCGTCTACCAATACCCGACTTGGTATTATGATGTCAGCGTTCCGGCAGGCCAAACGAT  5610
653     Y  N  Q  V  V  Y  Q  Y  P  T  W  Y  Y  D  V  S  V  P  A  G  Q  T  I   675
5611  TGAATTTAAATTCCTGAAAAAGCAAGGCTCCACCGTCACATGGGAAGGCGGCGCGAATCGCACCTTCACC  5680
676    E  F  K  F  L  K  K  Q  G  S  T  V  T  W  E  G  G  A  N  R  T  F  T   698
5681  ACCCCAACCAGCGGCACGGCAACGATGAATGTGAACTGGCAGCCTTAATAGGCACTTGCAAGGTAAGCAA  5750
699   T  P  T  S  G  T  A  T  M  N  V  N  W  Q  P                          713
5751  GCGGCTCCGGGTAGAGGCTCGGGGCCGCTTGTTACGTTATGTGGGGGAAAGGTGCTATAGGGTCTTGCCT  5820
5821  CCACACGCACGATTCTTAAGGTGTATTCACTGACCATAAAAGTACCCCAAGGATTTATCGAT            5882
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Figure 2. Nucleotide sequence of the DNA fragment containing the CGTase gene from *Bacillus circulans* A11. The start and stop codons are boxed. Putative promoter elements and the Shine–Dalgarno sequence are shaded. The signal sequence is double underlined. The sequence of the PCR product is underlined.

in Figure 2. Therein, the open reading frame for the 713 amino acid CGTase is found along with its upstream putative promoter elements and a Shine–Dalgarno sequence. The N-terminal amino acids of the CGTase from *B. circulans* A11 had previously been determined (unpublished), the first 27 amino acid residues, thus, constitutes the signal peptide. The deduced amino acid sequence was compared to those of the other CGTases, and was found to be highly similar to that of *Bacillus* sp. 1011 with sequence identities of 98.7% and less similar to that of *B. circulans* 251 with sequence identities of about 86% (data not shown).

2.2. Construction of chimeric cyclodextrin glucanotransferases

The amino acid sequence comparison reveals that CGTases with different product specificity have considerably similarity in amino acid sequence. Such similarity suggests the similarity in three-dimensional structure as well. How the CGTases with similar amino acid sequences and three-dimensional structures produce different ratios of cyclodextrins is interesting. It has been shown, by using domain-shuffling experiment between two CGTases with different specificity, that the N-terminal catalytic domains A/B confer the product specificity on the enzyme.[14] However, the domains A/B of about 400 amino acid residues (without signal peptide) are quite large compared to other domains. It is possible to dissect the domains further to uncover the peptide region that plays the key role in product specificity. To extend the similar study into the domains A/B, the domain-shuffling experiment was done by using two different CGTases, the α-CGTase from *Paenibacillus macerans* IAM1243,[15] which produces mainly α-CD and the β-CGTases from *B. circulans* A11, which produces mainly β-CD. A part of the domains A/B was also shuffled in the experiment.

Amino acid sequence comparison between the α- and β-CGTases reveals 68% identical amino acid residues, distributing along the entire sequence (Fig. 3). The β-CGTase is shorter by one amino acid residue. To facilitate the shuffling of the coding sequences of domains A/B, C, D, and E, three restriction sites, namely, *Xho*I, *Spe*I, and *Mfe*I, were introduced, respectively, at the hinge regions between domains. The positions of hinges were arbitrarily assigned based on the three-dimensional structures of CGTases from *Bacillus* sp. strain 1011[4] and *B. circulans* strain 251.[16] These new restriction sites were created by the unique site elimination mutagenesis, which led to changes of amino acid residues in the hinge regions. Lysine426 (numbering includes signal sequence) in β-CGTase and lysine427 in α-CGTase were replaced by serine, alanine525 in β-CGTase was replaced by serine, and valine614 in β-CGTase and valine615 in α-CGTase were replaced by leucine (Fig. 3). The positions of restriction sites were not coincided exactly with the hinge positions but they were within the hinge amino acid sequences, which were fairly conserved. In addition, the amino acid changes were designed to be more or less conservative.

The particular *Nde*I sites in the two CGTase coding sequences were also used in the domain-shuffling experiment (Fig. 3). The *Nde*I site separates domains A/B into two halves. The N-terminal half contains subdomain A1, domain B, and about half of subdomain A2, in which the conserved sequences I and II reside. The C-terminal half contains the rest of subdomain A2 in which the conserved sequences III and IV reside.

Mutagenesis of the α-CGTase-producing plasmid pVR300 and the β-CGTase-producing plasmid pVR328 finally resulted in mutant plasmids containing the desired three restriction sites, which were designated as pVR341 and pVR342, respectively. *E. coli* JM109 cells containing the pVR341 and pVR342 were tested positive for dextrinizing activity. However, the dextrinizing activity by pVR341 was considerably lower than that of the wild-type pVR300. The pVR342 and the wild-type pVR328 gave comparable dextrinizing activity (Fig. 4).

The DNA fragments corresponding to the CGTase domains and the *Nde*I-*Xho*I fragment were then exchanged between the two genes. Figure 6 shows all possible combinations of the DNA fragments exchanged between pVR341 and pVR342. This resulted in 30 chimeric CGTases, which could be divided into two groups, namely, the pVR300 series and pVR328 series, depending on whether the N-terminal portions were from α-CGTase in pVR300 or β-CGTase in pVR328 (Fig. 6).

2.3. Dextrinizing and cyclodextrin-forming activities of the chimeric CGTases

E. coli JM109 cells containing the chimeric CGTase genes were tested for dextrinizing activity on Luria-Bertani (LB) agar plates containing 1% soluble starch (Fig. 4). For pVR300 series, only three chimeras, pVR349, 351, and 357, retained their dextrinizing activity but the activity was lower than the pVR341. For pVR328 series, the chimeras pVR346, 348, 350, 354, 364, 380, 383, 384, and 385 were active in dextrinization. Among these, the pVR346, 364, 380, 384, and 385 showed lower dextrinizing activity than the pVR342. The dextrinizing activity is also summarized in Figure 6.

All the chimeric constructs were then tested for their cyclodextrin-forming activity by incubating the crude enzyme preparation with the soluble starch solution. The mixture of CDs thus formed was analyzed by HPLC. The percentages of CDs formed were calculated from the peak areas in HPLC profiles. The HPLC profiles from the chimeras that were able to produce functional CGTases are shown in Figure 5. The product ratios are summarized in Figure 6. The total amount

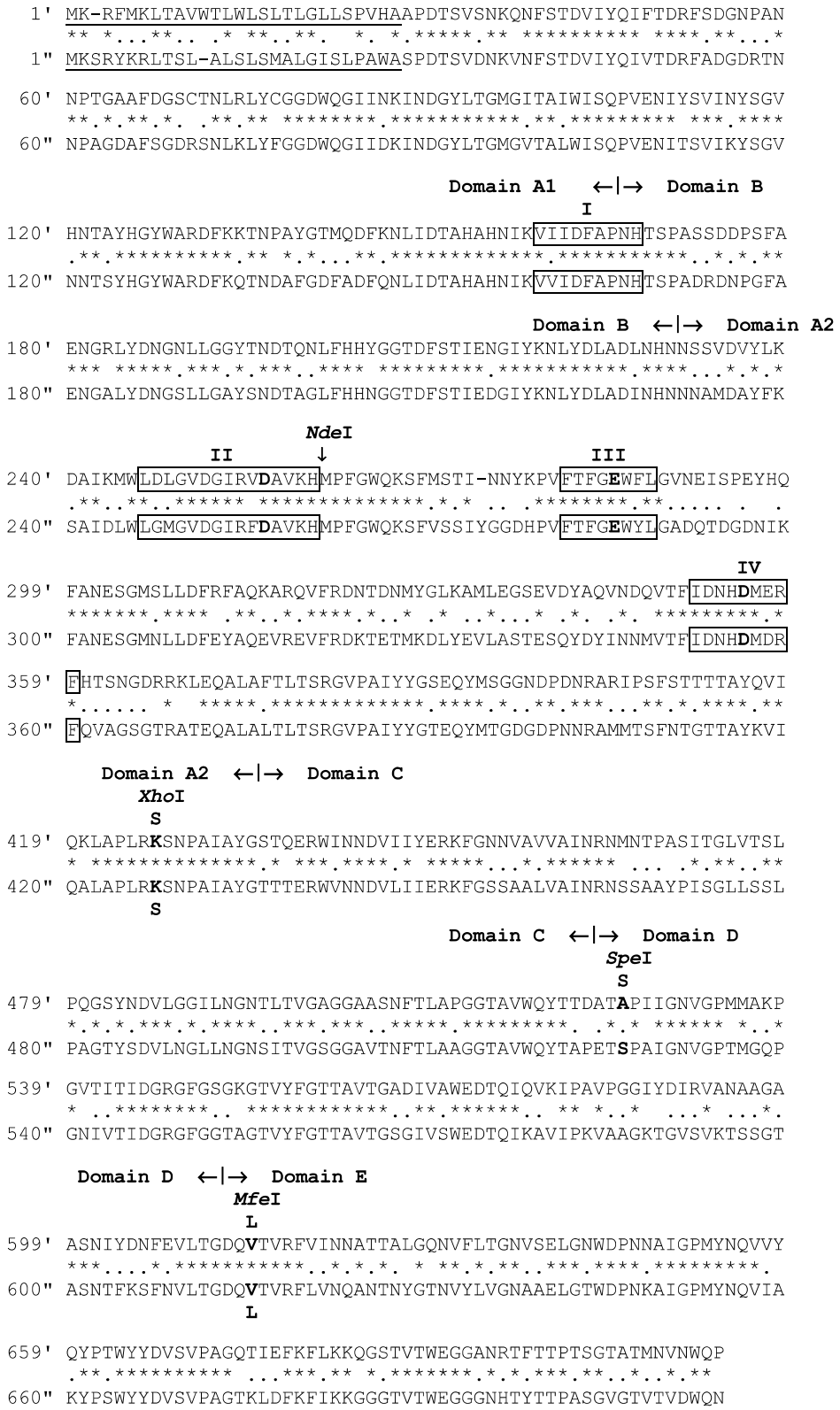


Figure 3. Amino acid sequence comparison between α- and β-CGTases. The amino acid sequences with signal sequences are aligned. The upper and lower amino acid sequences are β-CGTase and α-CGTase, respectively. The creation of *Xho*I, *Spe*I, and *Mfe*I restriction sites by mutagenesis results in the changes of the wild-type amino acid residues. The mutated amino acid residues are indicated above and/or below the corresponding wild-type amino acid residues. The signal sequences are underlined. The conserved sequences among enzymes in the α-amylase family are boxed. The catalytic residues, Asp, Glu, and Asp in conserved regions II, III, and IV, respectively, are bold-typed. The domains and the positions of restriction enzymes involved are indicated. The numbering used herein includes the signal peptide.

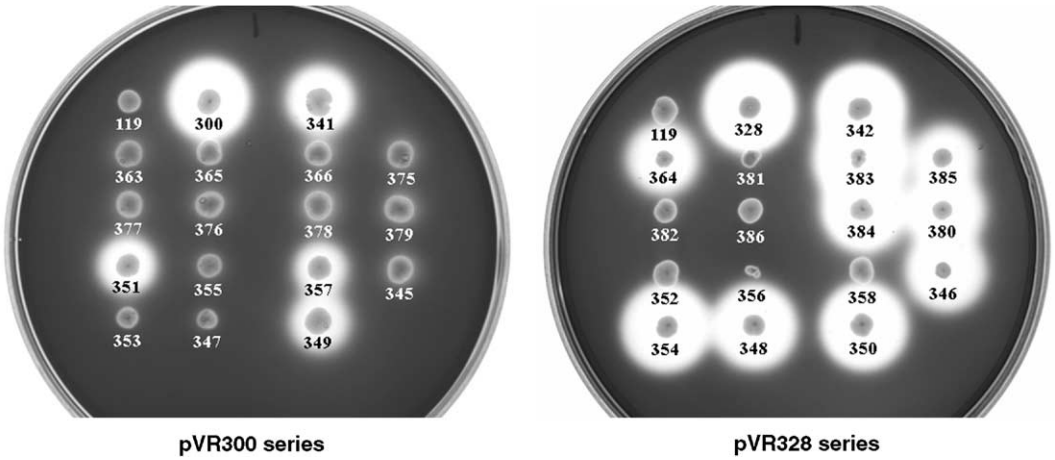


Figure 4. Dextrinizing activity test for the chimeric constructs. JM109 cells harboring the chimeric constructs were grown on LB agar plates containing 1% soluble starch for 2 days at 37 °C. Iodine reagent was poured onto the plates. Clear zone surrounding the colonies indicates starch-degrading activity.

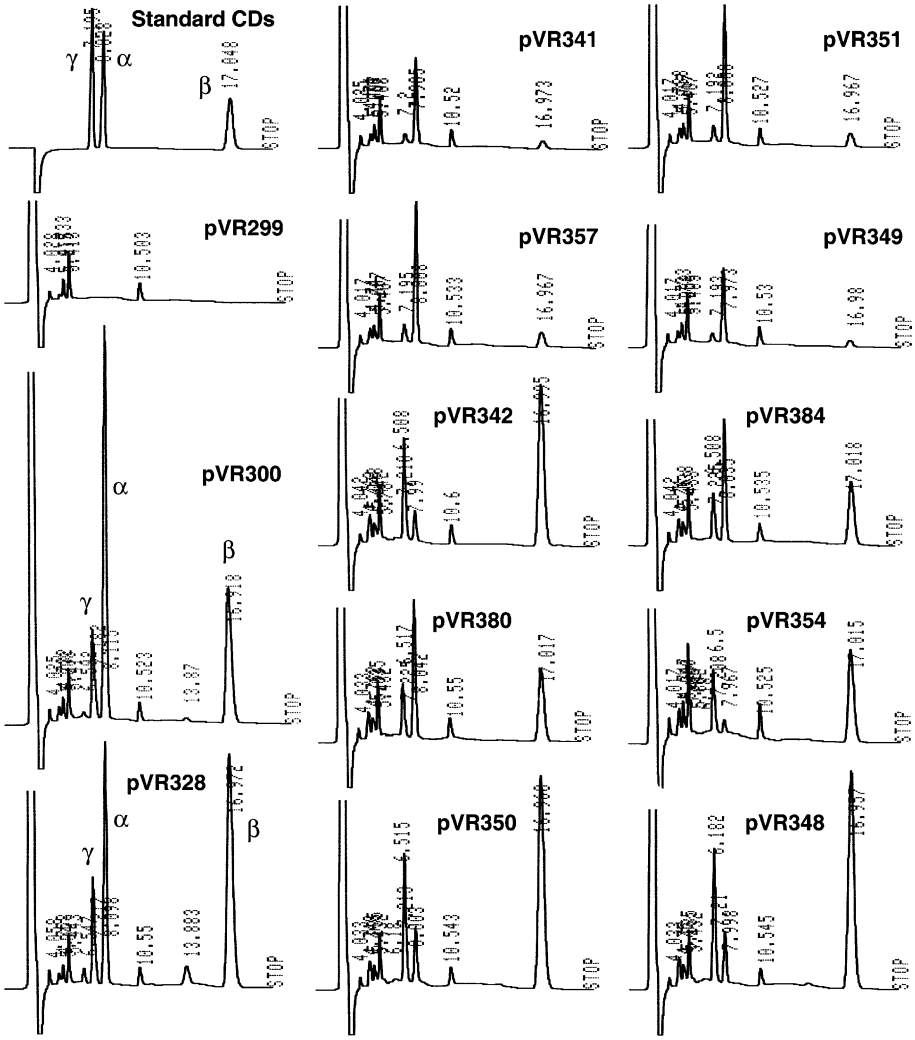
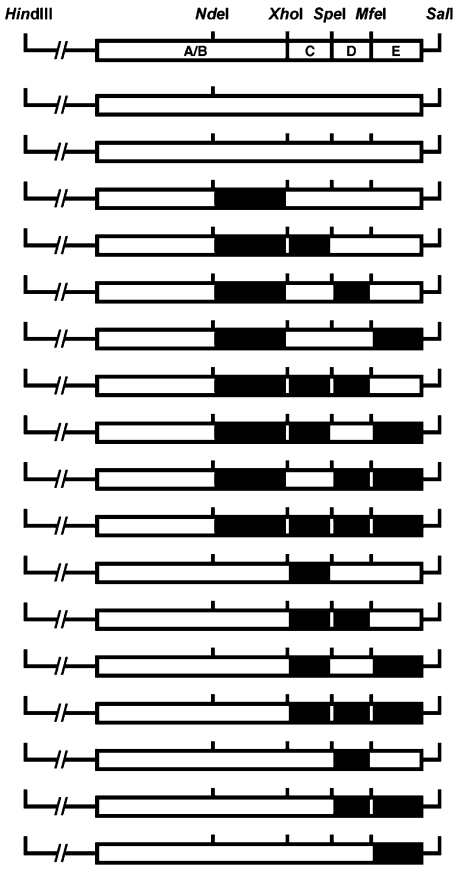


Figure 5. HPLC analysis of cyclodextrins formed by the chimeric CGTases. Representative HPLC profiles of the restriction site mutants and the chimeras actively produced CDs are shown. The control plasmid, pVR299, was a pUC119 derivative containing the PCR product of the CGTase gene from *Bacillus circulans* A11. The profile from pVR354 was obtained using double amount of sample. The identity of peak eluted at 13.88 min is not known.



pVR300 series	Dextrinizing activity/ CD ratio (α:β:γ)	Total CDs produced (%)	pVR328 series	Dextrinizing activity/ CD ratio (α:β:γ)	Total CDs produced (%)
300	+ / 58:29:13	100.0	328	+ / 32:55:13	100.0
341	+ / 75:14:11	7.7	342	+ / 7:70:23	49.2
363	− / −	−	364	+ / −	−
365	− / −	−	381	− / −	−
366	− / −	−	383	+ / −	−
375	− / −	−	385	+ / −	−
377	− / −	−	382	− / −	−
376	− / −	−	386	− / −	−
378	− / −	−	384	+ / 44:40:16	34.8
379	− / −	−	380	+ / 45:39:15	39.4
351	+ / 76:15:9	21.3	352	− / −	−
355	− / −	−	356	− / −	−
357	+ / 78:13:9	24.1	358	− / −	−
345	− / −	−	346	+ / −	−
353	− / −	−	354	+ / 5:70:25	14.4
347	− / −	−	348	+ / 8:70:22	71.8
349	+ / 78:12:10	12.0	350	+ / 9:71:20	74.0

Figure 6. Summary of the chimeric cyclodextrin glucanotransferase gene constructs, their dextrinizing and cyclodextrin-forming activities. Open box represents the CGTase gene from either *Paenibacillus macerans* IAM1243 in pVR300 or *B. circulans* A11 in pVR328 and close box represents the corresponding DNA sequence shuffling between the two plasmids. Working restriction sites used in the shuffling experiment are indicated.

of CDs formed by each chimera was also calculated as percentage of that of the wild-type CGTase.

The results showed that the replacement of two (K427S/V615L) and three amino acid residues (K426S/A525S/V614L), respectively, at the domain junctions in α- and β-CGTase, which accompanied the introduction of three new restriction sites, reduced the CD-forming activity of the mutants. For the mutant α-CGTase in pVR341, the total CDs obtained were decreased to 7.7% to that of the wild-type α-CGTase. The proportion of α-CD was increased while that of β-CD was decreased. For the mutant β-CGTase in pVR342, the total CDs obtained were decreased to 49.2%. The proportion of α-CD was decreased while those of β- and γ-CDs were increased. All other chimeras that produced active enzymes also gave lower amount of CDs as compared to the wild-type CGTases. Interestingly, chimeras pVR364, 383, 385, and 346 exhibited only starch-degrading activity. When the dextrinizing activity in Figure 4 was compared with the percentage of CDs obtained in Figure 6, it indicated that the chimeric enzymes including the pVR342 in the pVR328 series exhibited more amylolytic activity than the wild-type pVR328, particularly the pVR354.

3. Discussion

With the drawback in producing a mixture of CDs, new CGTases, which can produce a single CD product, have been sought. With the advent of amino acid sequence comparison among the CGTases and the detailed three-dimensional structures of the enzymes, scientists have gained more insight into the structure and function of the enzymes. The attempt to engineer the new enzymes with desired specificity has been made.[2,10,12] By examining the differences in the amino acid sequences and structures among CGTases, several amino acid residues or segments have been predicted to be involved in the product specificity and tested by using mutagenesis approach.[2] Some of the mutants have altered product ratios. However, none has succeeded in engineering the desired CGTase, which produces only one CD product. In addition, the amino acid changes usually result in the reduction of the CGTase activity, particularly, the total amount of CDs produced. More detailed studies of the CGTase are still further needed for the protein engineering of the enzyme.

In this research, we have cloned a β-CGTase from *B. circulans* A11 and along with the α-CGTase from

P. macerans IAM1243, created two series of chimeric CGTases in order to locate the amino acid sequence segment that governs the product specificity. The chimeric construction of CGTases had been done before by Fujiwara et al.[14] They constructed a series of chimeric CGTases by shuffling the domains A/B, C, D, and E between the two different CGTases and showed that the domains A/B were essential from the cyclization characteristics of the CGTases. By using the natural occurring *Nde*I sites at about the center of the domains A/B in the two CGTase genes, we further extended the study into the domains A/B. The shuffling of the DNA fragments corresponding to the domains between the two genes made use of three unique restriction sites, *Xho*I, *Spe*I, and *Mfe*I, created by mutagenesis at the hinge regions between the domains. Although the hinge regions are usually considered less important for the enzyme activity, in our experiment the amino acid changes that accompany the mutagenesis in pVR341 and 342 have deleterious effect on the enzyme activity, both the starch-degrading and the CD-forming activities as shown in Figures 4 and 6. Whichever amino acid substitutions affect the enzyme activity have not yet been determined. Moreover, the CD-ratios of both mutants change significantly. The reasonable explanation is that the amino acid substitutions have slightly changed the overall domain arrangement of the enzyme. Nevertheless, the changes are advantageous to the experiment because the two mutant CGTases gain more difference in product specificity, that is, the α- and β-CGTases produce higher proportions of α- and β-CDs, respectively. This is probably the first time that the correct fold at the hinge regions between the protein domains is shown experimentally to be important for the activity of the CGTase.

Thirty chimeras between the α-CGTase in pVR341 and β-CGTase in pVR342 were constructed and assayed for their activities. Eighteen chimeras are totally inactive, four chimeras have only dextrinizing activity, and eight chimeras are capable of degrading starch and producing CDs (Fig. 6). Most of the chimeras in pVR300 series are inactive. The three active chimeras, pVR351, 357, and 349, retain the cyclization characteristics of pVR341. The presence of domains C, E, and the combination of both from the counterpart pVR342 are acceptable, but not the *Nde*I-*Xho*I fragment, domain D, and its combination with other domains. The chimeras in pVR328 can tolerate better the presence of the domains from pVR341. The presence of domain C from pVR341 is unacceptable except that domains D and E from the same lineage are both presented, for example, pVR346 that exhibits only amylolytic activity. The presence of the counterpart *Nde*I-*Xho*I fragment or its combination with either domain D or domain E results in enzymes with only amylolytic activity, for example, pVR364, 383, and 385. The chimeras that contain domains D and E or both are active in dextrinization and CD-forming, and retain the cyclization characteristics of the parental pVR342, for example, pVR354, 348, and 350.

The most interesting chimeras in the pVR328 series are pVR384 and 380, which contain the counterpart *Nde*I-*Xho*I fragment, domain D and domain E, with or without domain C. They are active in dextrinization and CD-forming with altered specificity. They are in fact α-CGTases but do not possess the cyclization characteristics of pVR341. By comparing the characteristics of these two chimeras with those that contain the counterpart domains C, D, and/or E (pVR346, 354, 348, and 350), it is fair to conclude that the *Nde*I-*Xho*I fragment, at least for our β-CGTase, contains the determinant for product specificity, albeit the chimera with only such fragment (pVR364) can only degrade starch.

The overall result supports the conclusion made previously by Fujiwara et al.[14] that the domains A/B is essential for product specificity. We further limit that it is the C-terminal half of domains A/B that determines the product ratio. By examining the structure of the enzyme (Fig. 7), the C-terminal half of domains A/B constitutes the binding subsites +1 at His233 (numbering without signal sequence) to −3 at Asp371 opposite the domain B where the subsite −6 and −7 at Ser145 reside.[17] Together they form the active center for the two catalytic residues, Asp229 and Glu257, and probably the entrance for the oligosaccharide substrate. Domain E lies close to both the C-terminal half of domains A/B and domain B. Since domain E is a raw starch-binding domain, its proximity to the catalytic domain is thus important for the catalytic domain to gain access to

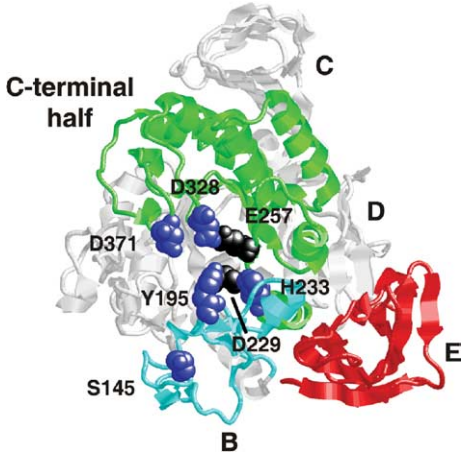


Figure 7. The cartoon structure of CGTase showing the various domains, substrate binding sites, and the amino acid residues involved in substrate binding and catalysis. The C-terminal half of domains A/B, domain B, and domain E are shown in green, magenta, and red, respectively. The amino acid residues surrounding the substrate binding subsites and the two catalytic residues are shown in blue and black, respectively.

the substrate. The results in this study have shown that domains C and D have strong influence on the enzyme activity. Suitable combinations of domains C, D, and E are important for the functionality of the enzyme. It is thus the combinations that lead to the correct orientation and proximity of domain E against domains A/B that give rise to functional CGTase. Therefore, the function of domains C and D are likely to position domain E properly in the enzyme.

4. Experimental

4.1. Bacterial strains

B. circulans A11 was used as a source of chromosomal DNA for cyclodextrin glucanotransferase gene cloning. *E. coli* BMH 71-18 *mut*S BMH 71-18 [Δ(*lac-proAB*), *thi*, *supE*/F′ *lacI*^{q}, *lacZ*ΔM15, *proA*$^+$*B*$^+$] was used in unique site elimination mutagenesis procedure. *E. coli* JM109 [*recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17*, *supE44*, *relA1*, Δ(*lac-proAB*)/F′ *traD36*, *proA*$^+$*B*$^+$, *lacI*^{q}, *lacZ*ΔM15] was used as a host for the cloning and the preparation of crude CGTase for activity assay.

4.2. Materials

Restriction endonucleases, T7 DNA polymerase, T4 kinase, bacterial alkaline phosphatase, *Taq* DNA polymerase, and Ligation High were from Takara Biomedicals and Toyobo Biochemicals.

4.3. Plasmids

A pGEM®-T vector was used for the cloning of the CGTase-specific PCR fragment amplified from the chromosomal DNA of *B. circulans* A11. A pUC119 vector was used for general cloning. A plasmid pDS10 containing a CGTase gene from *P. macerans* IAM1243 was a gift from Mr. Toshiya Takano.[15] The 2517 base pair *Sph*I-*Bam*HI DNA fragment containing the CGTase gene from pDS10 was subcloned into pUC119 and the derivative was designated as pVR300.

4.4. Preparation of a cyclodextrin glucanotransferase gene-specific DNA probe

The cyclodextrin glucanotransferase gene-specific DNA probe was amplified from the chromosomal DNA of *B. circulans* A11, using a pair of degenerate primers, 5′TGGATYTCNCAGCCDGTNGAAAA3′ and 5′CATRTCATGRTTRTCDATRAA3′ (Amersham Pharmacia Biotech), designed from the relatively homologous sequences among the CGTase amino acid sequences of *Bacillus* sp. 1011,[18] *Bacillus* sp. 38-2,[19] *B. licheniformis*,[20] *Brevibacillus brevis* (GenBank Accession AF011388), *B. circulans* 251,[5] *Bacillus Q* CK104 (GenBank Accession L25256), *Bacillus* sp. KC201,[21] *P. macerans* IAM1243,[15] and *B. stearothermophilus*.[14] The PCR reaction was carried out in 50 μL in the presence of 1 μg of the chromosomal DNA from *B. circulans* A11 and 1 μM of each primer. The PCR was started with a cycle of 94 °C for 6 min, 45 °C for 2 min, and 72 °C for 3 min, followed by 25 cycles of 94 °C for 1 min, 45 °C for 2 min, and 72 °C for 3 min and ended with a cycle of 94 °C for 1 min, 45 °C for 2 min, and 72 °C for 13 min. A single PCR product of about 850 base pairs was obtained, cloned into the pGEM®-T vector (Promega) and sequenced. The nucleotide sequence was determined to be that of CGTase by sequence comparison. The PCR product was then used as probe for the cloning of CGTase gene.

4.5. Cloning of the cyclodextrin glucanotransferase gene from *B. circulans* A11

The cyclodextrin glucanotransferase gene from *B. circulans* A11 was cloned using conventional DNA cloning technique.[22] Chromosomal DNA from *B. circulans* A11, 10 μg per reaction, was digested with *Pvu*II, *Xba*I, *Eco*RI, *Pst*I, *Sal*I, *Sph*I, *Bam*HI, and *Sma*I to completion and subjected to 0.8% agarose gel electrophoresis. Southern blot hybridization was carried out using a PCR DNA fragment as a probe to locate the DNA fragments containing the CGTase gene. Hybond™-N+ membrane and Gene Images™ for hybridization (Amersham Pharmacia Biotech) were used. Hybridization was done according to the company's procedure. The DNA fragments about the sizes where the hybridized DNA fragments were located from *Sph*I and *Bam*HI digestion reactions were eluted from the agarose gel pieces and cloned into a pUC119. Colony hybridization was used to identify the clones containing CGTase sequence. The clones were subjected to restriction mapping and DNA sequencing.

4.6. Mutagenesis

Single-stranded plasmids were prepared from pVR328, a pUC119 plasmid carrying the β-CGTase gene from *B. circulans* A11, and pVR300, a pUC119 plasmid carrying the α-CGTase gene from *P. macerans* IAM1243 using a protocol described by Viera and Messing.[23] Six mutagenic oligonucleotides as shown in Figure 8 were purchased from Espec Oligo Service Corp., Tokyo. Mutagenic oligonucleotides A, B, and C were used to create *Xho*I, *Spe*I, and *Mfe*I sites in pVR328, respectively. Mutagenic oligonucleotides D, E, and F were used to create the same restriction sites in pVR300.

The unique site elimination mutagenesis was carried out as follows.[24] Each mutagenic oligonucleotide was

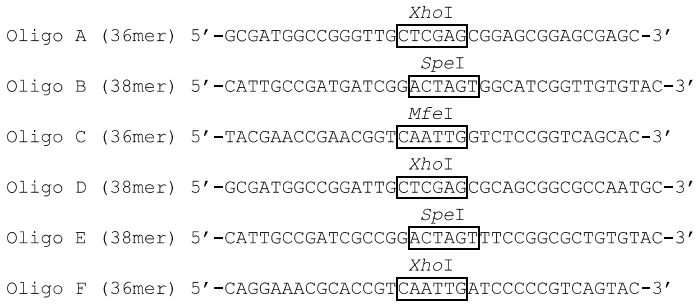


Figure 8. Oligonucleotides used in unique site elimination mutagenesis procedure. The restriction recognition sites are boxed.

5′-phosphorylated using T4 polynucleotide kinase in the presence of 1 mM ATP. Approximately 10 pmol each of the three kinased oligonucleotides (oligos A, B, and C or oligos D, E, and F) along with 10 pmol of kinased mutagenic *Sca*I oligonucleotide, 5′TCTGTG-ACTGGTGAATACTCAACCAAGTC3′, were annealed to 0.2 pmol of the single-stranded plasmids in a volume of 10 μL at 95 °C for 1 min and slowly cooled to room temperature. The extension of the oligonucleotides and ligation reactions were then carried out by adding 6 μL of enzyme mixture containing 1.5 μL T4 DNA polymerase buffer, 2 units of T7 polymerase, 5 units of T4 ligase, 1.5 μL of 2.5 mM dNTP, and 1.5 μL of 10 mM ATP. The reactions were incubated for 2 h at 37 °C. The mixture was transformed into an *E. coli* BMH 71-18 *mut*S, which was then cultured for an overnight in the presence of 100 μg/mL ampicillin. A mixture of plasmids were prepared, digested with *Sca*I and retransformed into *E. coli* JM109. Individual plasmids were prepared from colonies and screened for the presence of three new restriction sites. Two plasmids, pVR333 for α-CGTase and pVR334 for β-CGTase, were obtained. The DNA sequences around the new restriction sites were sequenced to assure that no mutations other than those intended were found. The DNA fragments containing the desired restriction sites were subcloned into the same sites in the original plasmids pVR300 and pVR328. The final constructs were pVR341 for α-CGTase and pVR342 for β-CGTase.

4.7. Construction of the chimeric CGTase genes

For the DNA exchange between pVR341 and pVR342, each plasmid was digested with the appropriate restriction enzymes, for example, *Nde*I and *Xho*I for the exchange of the *Nde*I-*Xho*I fragment, the digests were subjected to agarose gel electrophoresis to separate the DNA fragments. The eluted DNA fragment corresponding to the domain was then ligated to the same sites in the eluted counterpart plasmid. Likewise, all other chimeras with all possible combinations of the domains were constructed. The correct construction of the chimeric plasmids was confirmed by restriction analysis because the restriction maps of α- and β-CGTase genes were different.

4.8. Dextrinizing activity

The chimera clones were tested for dextrinizing or starch-degrading activity. *E. coli* JM109 cells, each containing chimeric plasmid, were grown on LB agar plates containing 1% soluble starch for 2 days at 37 °C in the presence or absence of 0.5 mM IPTG. Iodine solution (0.02% I_2 in 0.27% KI) was poured onto the plates. Dextrinizing-positive clones showed surrounding clear zones.

4.9. Cyclodextrin producing activity

Upon culturing, the CGTases produced by the JM109 transformants were secreted into the medium, and the cell culture medium was used as a source of crude enzyme for assaying the CD-forming activity. All dextrinizing inactive chimeras were also tested. To avoid differences in assay condition, all of the enzymes were prepared and assayed at the same time. The transformants were grown in LB medium at 37 °C overnight and pelleted by centrifugation. Each supernatant liquid of 200 μL was incubated with 0.5 mL of 1% soluble starch at 37 °C for 6 h. The reaction was stopped by boiling for 10 min. The reaction was clarified by centrifugation and the supernatant liquid was treated for an overnight with about 30 units of glucoamylase to digest the remaining starch. After stopping the glucoamylase reaction by boiling, the reaction was again clarified by centrifugation. The supernatant liquid of 100 μL was analyzed by high performance liquid chromatography for cyclodextrin content using a reverse phase YMC-Pack ODS-A C18 column (250 × 4.6 mm ID) and RI detector. The eluent was 5% MeOH in water and the flow rate of 1 mL/min was used.

The cyclodextrin ratio was calculated as percents from the areas under the peaks of α-, β-, and γ-cyclodextrins. The total cyclodextrin produced by the chimeric enzyme was also expressed as percentage of that of the wild-type CGTase.

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