CHAPTER III
RESULTS

3.1. Comparison of the amino acid sequence of the CGTase from *Bacillus circulans* A11 with those of thermostable CGTases and the design of mutagenic primers

CGTases from different sources have different characteristics in terms of activity, optimum temperature, thermostability, substrate specificity, and some local structures. The differences lie on the primary amino acid sequence, which dictates the higher-level structure of the enzyme. It is believed that one could simulate one enzyme with the other by mimicking the primary structure of the former enzyme. In this study, I attempted to create a thermophilic CGTase from the mesophilic one from *Bacillus circulans* A11 (A11 CGTase). The amino acid sequences from different thermophilic organisms were downloaded from the GenBank for amino acid sequence comparison. They were CGTases from *Thermoanaerobacterium thermosulfurigenes* EM1 (Tonkova, 1998) *Thermoanaerobacter* sp. ATCC 53627, *B. stearothermophilus* NO2 and *B. stearothermophilus* ET1 (Chung *et al.*, 1998), which had temperature optima of 80-85, 90 and 80 °C, respectively. Of these, the crystal structures of CGTases from *T. thermosulfurigenes* EM1 (Knegtel *et al.*, 1996) and *B. stearothermophilus* (Kubota M, Matsuura Y, Sakai S, Katsube Y, PDB 1CYG, unpublished) were known.

The amino acid sequence of CGTase from *B. circulans* A11 was then compared to those of the thermostable CGTases using clustalX (Thompson *et al.*, 1999). The result is shown in Fig. 3.1. The result shows moderate homology (~70%) among the five CGTases as several homologous segments are seen scattering all over the entire amino acid sequences. There are, however, three major and one minor regions in the A11 CGTases that are different from those of thermostable CGTases. The four regions (I, II, III and IV) consist of amino acid residues at positions 89-94, 265-271, 333-339, and 538-540 (A11 CGTase numbering), respectively. The first

three regions reside in the catalytic domain A/B while the fourth region is located in domain D whose function is not fully unveil. The fourth region is likely not involved in the thermostability of the enzyme since it is found also in some thermostable CGTases.

The primers, corresponding to the three different amino acid regions in A11 CGTase, were then designed. The designed primers mimic the amino acid residues of the thermostable CGTases. Two primers, A and B, were designed for region I mutagenesis, because there were variation in amino acid sequences among the four thermostable CGTases. Primers C and D were designed for region II and III mutagenesis, respectively. Primer A and B introduced base substitutions/addition and substitutions/deletion, respectively. Primer C and D provided only base substitutions (Fig. 3.2). Amino acid substitutions were made in favor of the amino acid sequence in CGTase from thermostable enzyme. Upon mutagenesis, the amino acid sequences in CGTase from *B. circulans* A11 would be changed to DSTFGGS, DASGS, VDPNNHY, and YIGEGDT in regions I_A, I_B, II, and III, respectively. For the screening of mutants, primers A, and D were designed to create restriction sites, *Bam*HI and *Hin*dIII, respectively, in the mutant plasmids. Primers B and C were both designed to create the same restriction site, *Sal*I, in the mutant plasmids.

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ATCC53627       APDTSVSNVVNYSTDVIYQIVTDRFLDGNPSNNPTGDLYDPTHTSLKKYFGGDWQ 55
1CIU            ASDTAVSNVVNYSTDVIYQIVTDRFVDGNTSNNPTGDLYDPTHTSLKKYFGGDWQ 55
A11             APDTSVSNKQNFSTDVIYQIFTDRFSDGNPANNPTGAAFDGSCTNLRLYCGGDWQ 55
ET1             ---AGNLNKVNFTSDIVYQIVVDRFVDGNTSNNPSGSLFSSGCTNLRKYCGGDWQ 52
No2             ---AGNLNKVNFTSDVVYQIVVDRFVDGNTSNNPSGALFSSGCTNLRKYCGGDWQ 52
                   :.  *  *:::*::***..*** ***.:***:*  :.   *.*: * *****

                                                  IA/IB
                                                <------>
ATCC53627       GIINKINDGYLTGMGITAIWISQPVENIYAVLPDSTFGGSTSYHGYWARDFKKPN 110
1CIU            GIINKINDGYLTGMGVTAIWISQPVENIYAVLPDSTFGGSTSYHGYWARDFKRTN 110
A11             GIINKINDGYLTGMGITAIWISQPVENIYSVINYSGVHN-TAYHGYWARDFKKTN 109
ET1             GIINKINDGYLTEMGVTAIWISQPVENVFAVMNDA--DGSTSYHGYWARDFKKTN 108
No2             GIINKINDGYLTDMGVTAIWISQPVENVFSVMNDA--SGSASYHGYWARDFKKPN 108
                ************ **:***********:::*:  :   . ::**********:.*
                                          primerA DSTFGGS
                                          primerB DA--SGS

ATCC53627       PFFGSFTDFQNLIATAHAHNIKVIIDFAPNHTSPASETDPTYGENGRLYDNGVLL 165
1CIU            PYFGSFTDFQNLINTAHAHNIKVIIDFAPNHTSPASETDPTYAENGRLYDNGTLL 165
A11             PAYGTMQDFKNLIDTAHAHNIKVIIDFAPNHTSPASSDDPSFAENGRLYDNGNLL 164
ET1             PFFGTLSDFQRLVDAAHAKGIKVIIDFAPNHTSPASETNPSYMENGRLYDNGTLI 160
No2             PFFGTLSDFQRLVDAAHAKGIKVIIDFAPNHTSPASETNPSYMENGRLYDNGTLL 160
                  :*:: **:.*: :***:.**************. :*:: ********* *:

ATCC53627       GGYTNDTNGYFHHYGGTNFSSYEDGIYRNLFDLADLDQQNSTIDSYLKAAIKLWL 220
1CIU            GGYTNDTNGYFHHYGGTDFSSYEDGIYRNLFDLADLNQQNSTIDSYLKSAIKVWL 220
A11             GGYTNDTQNLFHHYGGTDFSTIENGIYKNLYDLADLNHNNSSVDVYLKDAIKMWL 219
ET1             GGYTNDTNSYFHHNGGTTFSNLEDGIYRNLFDLADFNHQNQFIDKYLKDAIKLWL 215
No2             GGYTNDANMYFHHNGGTTFSSLEDGIYRNLFDLADLNHQNPVIDRYLKDAVKMWI 215
                ******::  *** *** **. *:***:**:****::::*  :* *** *:*:*:

                                                        II
                                                      <------>
ATCC53627       DMGIDGIRMDAVKHMAFGWQKNFMDSILSYRPVFTFGEWYLGTNEVDPNNTYFAN 275
1CIU            DMGIDGIRLDAVKHMPFGWQKNFMDSILSYRPVFTFGEWFLGTNEIDVNNTYFAN 275
A11             DLGVDGIRVDAVKHMPFGWQKSFMSTINNYKPVFTFGEWFLGVNEISPEYHQFAN 274
ET1             DMGIDGIRMDAVKHMPFGWQKSFMDEVYDYRPVFTFGEWFLSENEVDSNNHFFAN 270
No2             DMGIDGIRMDAVKHMPFGWQKSLMDEIDNYRPVFTFGEWFLSENEVDANNHYFAN 270
                *:*:****:******.*****.:*. : .*:*********:*. **:. :   ***
                                                    primerC VDPNNHY

ATCC53627       ESGMSLLDFRFAQKVRQVFRDNTDTMYGLDSMIQSTAADYNFINDMVTFIDNHDM 330
1CIU            ESGMSLLDFRFSQKVRQVFRDNTDTMYGLDSMIQSTASDYNFINDMVTFIDNHDM 330
A11             ESGMSLLDFRFAQKARQVFRDNTDNMYGLKAMLEGSEVDYAQVNDQVTFIDNHDM 329
ET1             ESGMSLLDFRFGQKLRQVLRNNSDDWYGFNQMIQDTASAYDEVIDQVTFIDNHDM 325
No2             ESGMSLLDFRFGQKLRQVLRNNSDNWYGFNQMIQDTASAYDEVLDQVTFIDNHDM 325
                ***********.** ***:*:*:*  **:. *::.:   *  : * *********

                      III
                    <------>
ATCC53627       DRFYTG-GSTRPVEQALAFTLTSRGVPAIYYGTEQYMTGNGDPYNRAMMTSFDTT 384
1CIU            DRFYNG-GSTRPVEQALAFTLTSRGVPAIYYGTEQYMTGNGDPYNRAMMTSFNTS 384
A11             ERFHTSNGDRRKLEQALAFTLTSRGVPAIYYGSEQYMSGGNDPDNRARIPSFSTT 384
ET1             DRFMADEGDPRKVDIALAVLLTSRGVPNIYYGTEQYMTGNGDPNNRKMMTSFNKN 380
No2             DRFMIDGGDPRKVDMALAVLLTSRGVPNIYYGTEQYMTGNGDPNNRKMMSSFNKN 380
                :**  . *. * :: ***. ******* ****:****:*..** **  :.**...
      primerD Y(N/I)(G/D)EGD(T/P)
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ATCC53627       TTAYNVIKKLAPLRKSNPAIAYGTQKQRWINNDVYIYERQFGNNVALVAINRNLS 439
1CIU            TTAYNVIKKLAPLRKSNPAIAYGTTQQRWINNDVYIYERKFGNNVALVAINRNLS 439
A11             TTAYQVIQKLAPLRKSNPAIAYGSTQERWINNDVIIYERKFGNNVAVVAINRNMN 439
ET1             TRAYQVIQKLSSLRRSNPALSYGDTEQRWINSDVYIYERQFGKDVVLVAVNRSLS 435
No2             TRAYQVIQKLSSLRRNNPALAYGDTEQRWINGDVYVYERQFGKDVVLVAVNRSSS 435
                * **:**:**:.**:.***::**  ::****.** :***:**::*.:**:**. .

ATCC53627       TSYYITGLYTALPAGTYSDMLGGLLNGSSITVSSNGSVTPFTLAPGEVAVWQYVS 494
1CIU            TSYNITGLYTALPAGTYTDVLGGLLNGNSISVASDGSVTPFTLSAGEVAVWQYVS 494
A11             TPASITGLVTSLPQGSYNDVLGGILNGNTLTVGAGGAASNFTLAPGGTAVWQYTT 494
ET1             KSYSITGLFTALPSGTYTDQLGALLDGNTIQVGSNGAVNAFNLGPGEVGVWTYSA 490
No2             SNYSITGLFTALPAGTYTDQLGGLLDGNTIQVGSNGSVNAFDLGPGEVGVWAYSA 490
                .   **** *:** *:*.* **.:*:*.:: *.:.*:.. * *..* ..** * :

                                                          IV
                                                        <-->
ATCC53627       TTNPPLIGHVGPTMTKAGQTITIDGRGFGTTAGQVLFGTTPAT---IVSWEDTEV 546
1CIU            SSNSPLIGHVGPTMTKAGQTITIDGRGFGTTSGQVLFGSTAGT---IVSWDDTEV 546
A11             DATAPIIGNVGPMMAKPGVTITIDGRGFGSGKGTVYFGTTAVTGADIVAWEDTQI 549
ET1             AESVPIIGHIGPMMGQVGHKLTIDGEGFGTNVGTVKFGNTVAS---VVSWSNNQI 542
No2             TESTPIIGHVGPMMGQVGHQVTIDGEGFGTNTGTVKFGTTAAN---VVSWSNNQI 542
                  . *:**::** * : *  :****.***:  * * **.*  .   :*:*.:.::

ATCC53627       KVKVPALTPGKYNITLKTASGVTSNSYNNINVLTGNQVCVRFVVNNATTVWGENV 601
1CIU            KVKVPSVTPGKYNISLKTSSGATSNTYNNINILTGNQICVRFVVNNASTVYGENV 601
A11             QVKIPAVPGGIYDIRVANAAGAASNIYDNFEVLTGDQVTVRFVINNATTALGQNV 604
ET1             TVTVPNIPAGKYNITVQTSGGQVSAAYDNFEVLTNDQVSVRFVVNNANTNWGENI 597
No2             VVAVPNVSPGKYNITVQSSSGQTSAAYDNFEVLTNDQVSVRFVVNNATTNLGQNI 597
                 * :* :. * *:* : .::.* .*  *:*:::**.:*: ****:***.*  *:*:

ATCC53627       YLTGNVAELGNWDTSKAIGPMFNQVVYQYPTWYYDVSVPAGTTIEF--IKKNGS- 653
1CIU            YLTGNVAELGNWDTSKAIGPMFNQVVYQYPTWYYDVSVPAGTTIQFKFIKKNGN- 655
A11             FLTGNVSELGNWDPNNAIGPMYNQVVYQYPTWYYDVSVPAGQTIEFKFLKKQGS- 658
ET1             YLVGNVHELGNWNTSKAIGPLFNQVIYSYPTWYVDVSVPEGKTIEFKFIKKDGSG 652
No2             YIVGNVYELGNWDTSKAIGPMFNQVVYSYPTWYIDVSVPEGKTIEFKFIKKDSQG 652
                ::.*** *****:..:****::***:*.***** ***** * **:*  :**:..

ATCC53627       TVTWEGGYNHVYTTPTSGTATVIVDWQP 681
1CIU            TITWEGGSNHTYTVPSSSTGTVIVNWQQ 683
A11             TVTWEGGANRTFTTPTSGTATMNVNWQP 686
ET1             NVIWESGSNHVYTTPTSTTGTVNVNWQY 680
No2             NVTWESGSNHVYTTPTNTTGKIIVDWQN 680
                .: **.* *:.:*.*:. *..: *:**
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Fig. 3.1. Amino acid sequence comparison of the various CGTases. The different amino acid sequences of CGTases from *Thermoanaerobacterium thermosulfurigenes* EM1 (1CIU), *Thermoanaerobacter* sp. ATCC 53627 (ATCC53627), *Bacillus circulans* A11 (A11), *Bacillus stearothermophilus* NO2 (No2) and *Bacillus stearothermophilus* ET1 (ET1) were aligned. Double arrows indicate the amino acid sequences of CGTase from *B. circulans* A11 that are different from those of thermostable CGTases. Numbering of the amino acid sequence starts at the N- terminal amino acid of each mature enzyme.

PRIMER A (47mer)

```
                                    BamHI
PRIMER          5' C GTG ATA AGC CGT GGA TCC GCC GAA GGT GGA ATC GTT GAT CAC GCT G 3'
MUTANT          3' G CAC TAT TCG GCA CCT AGG CGG CTT CCA CCT TAG CAA CTA GTG CGA C 5'
                                  ← S   G   G   F   T   S   D
WILD TYPE       3' G CAC TAT TCG GCA --- TAA TAC CTG CGG CCT CAT CAA CTA GTG CGA C 5'
                                    ← N   H   V   G   S   Y
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PRIMER B (41mer)

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                                    SalI
PRIMER          5' C GTG ATA AGC CGT --- --- CGA CCC GCT GGC ATC GTT GAT CAC GCT G 3'
MUTANT          3' G CAC TAT TCG GCA --- --- GCT GGG CGA CCG TAG CAA CTA GTG CGA C 5'
                                      ← S   G   S   A   D
WILD TYPE       3' G CAC TAT TCG GCA TAA TAC CTG CGG CCT --- CAT CAA CTA GTG CGA C 5'
                                  ← N   H   V   G   S       Y
```

PRIMER C (51mer)

```
                                                    SalI
PRIMER      5' GGA CTC GTT AGC GAA GTA ATG ATT GTT CGG GTC GAC CTC ATT GAC GCC AAG 3'
MUTANT      3' CCT GAG CAA TCG CTT CAT TAC TAA CAA GCC CAG CTG GAG TAA CTG CGG TTC 5'
                              ← Y   H   N   N   P   D   V
WILD TYPE   3' CCT GAG CAA TCG CTT AAC TAC CAT AAG GCC TGA TTA GAG TAA CTG CGG TTC 5'
                              ← Q   H   Y   E   P   S   I
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PRIMER D (56mer)

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                          HindIII
PRIMER      5' CCAGCGCCTGCTC AAG CTT CCG TG(T/G) GTC GCC CTC G(C/T)C G(T/A)T GTA GAAACGCTCCAT G 3'
MUTANT      3' GGTCGCGGACGAG TTC GAA GGC AC(A/C) CAG CGG GAG C(G/A)G C(A/T)A CAT CTTTGCGAGGTA C 5'
                                  ← T/P   D   G   E   G/D     N/I     Y
WILD TYPE   3' GGTCGCGGACGAG GTC GAA GGC   AGA   CAG CGG TAA   CGA     CCA   CAC CTTTGCGAGGTA C 5'
                                  ← R     D   G   N     S       T     H
```

Fig. 3.2. The design of oligonucleotides used in the USE mutagenesis procedure. The nucleotide sequences of wild type to be mutated are underlined. The newly created restriction sites are shaded.

3.2. Mutagenesis of CGTase gene from *Bacillus circulans* A11 using USE mutagenesis

The plasmid pVR328, carrying the CGTase gene from *B. circulans* A11, was used as a template for the USE mutagenesis procedure (Fig.2.2). The mutagenic primers A, B, C, and D along with the reference *Sca*I primer were used to mutate the CGTase gene at the three regions described above. The mutagenesis procedure generated a mixture of mutated plasmids. After removing the mostly wild type plasmids with *Sca*I digestion, the mutated plasmids were separated into individual clones by transformation. The plasmid clones were prepared, and screened for the presence of added restriction sites, *Bam*HI, *Sal*I, and *Hin*dIII. The positions of these sites were confirmed by restriction enzyme digestion and agarose gel electrophoresis (Fig. 3.3). Four mutated plasmids, pRS1A, 1B, 2 and 3, were obtained as shown in Fig. 3.4 with the restriction sites, *Bam*HI, *Sal*I, *Sal*I, and *Hin*dIII, respectively. It should be noted that the pRS3 were obtained after screening nine clones because the designed primer contained multiple nucleotides in several positions in order to create multiple mutated sequences. Two identical clones were selected after restriction digestion and DNA sequencing. Only one clone was chosen to represent the pRS3.

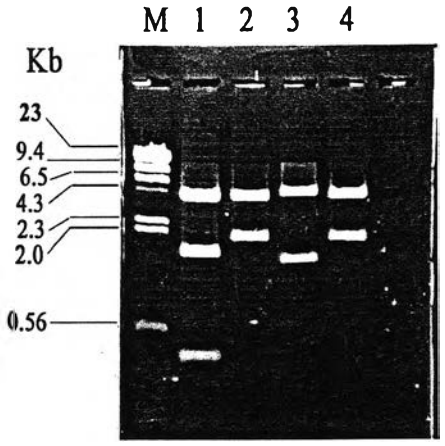


Fig. 3.3. Restriction digestion of pRS1A, 1B, 2, and 3. Lane M: λ/*Hin*dIII marker; lane 1: pRS1A digested with *Bam*HI, lane 2: pRS1B digested with *Sal*I, lane 3: pRS2 digested with *Sal*I, and lane 4: pRS3 digested with *Hin*dIII.

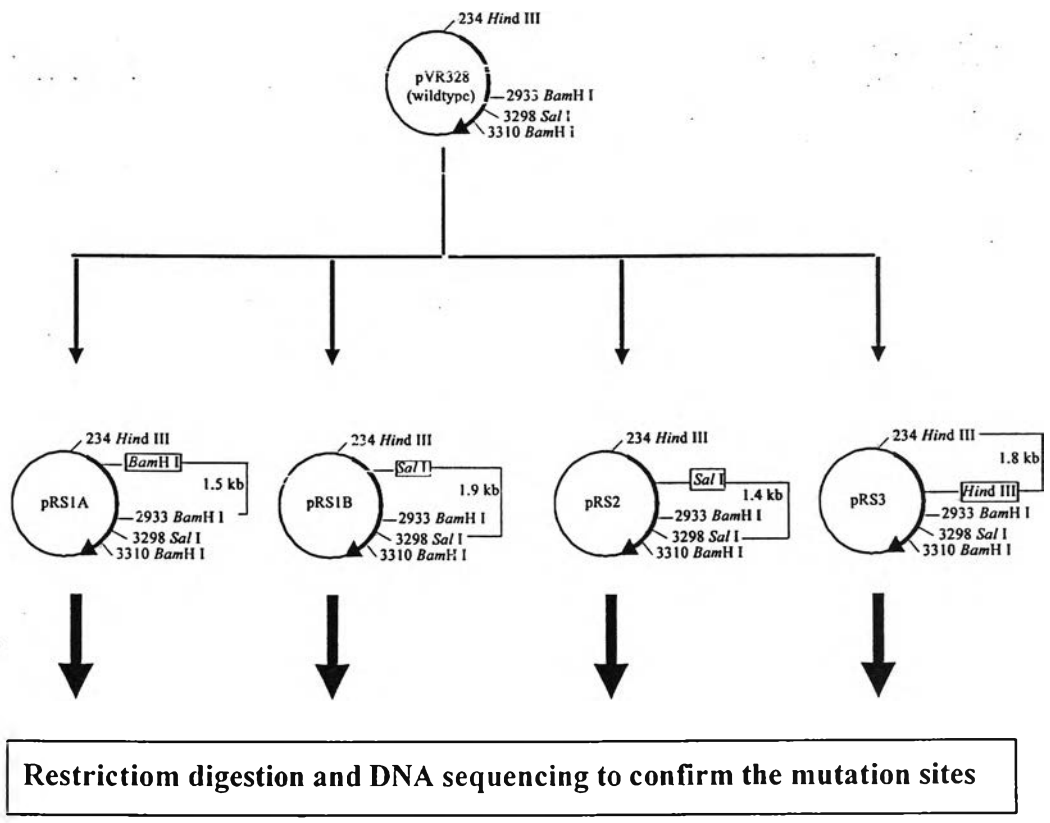


Fig. 3.4. The mutated plasmids, pRS1A, 1B, 2 and 3. The plasmids were processed to confirm the mutations by restriction digestion and DNA sequencing. The new restriction sites are boxed and the sizes of the DNA fragments are indicated.

3.3. DNA sequencing determination of the mutation regions

The precise DNA sequences around the mutation regions in pRS1A, 1B, 2 and 3 were determined using the ABI Prism Big Dye Terminator Cycle Sequencing. The four mutants had the nucleotide sequences as designed (Fig. 3.5). Nucleotide sequences between the two restriction sites bordering the mutation regions were also checked to make certain that there were no additional mutations. The mutation regions were subsequently subcloned using the two border restriction sites into the wild type plasmid replacing the wild type sequences.

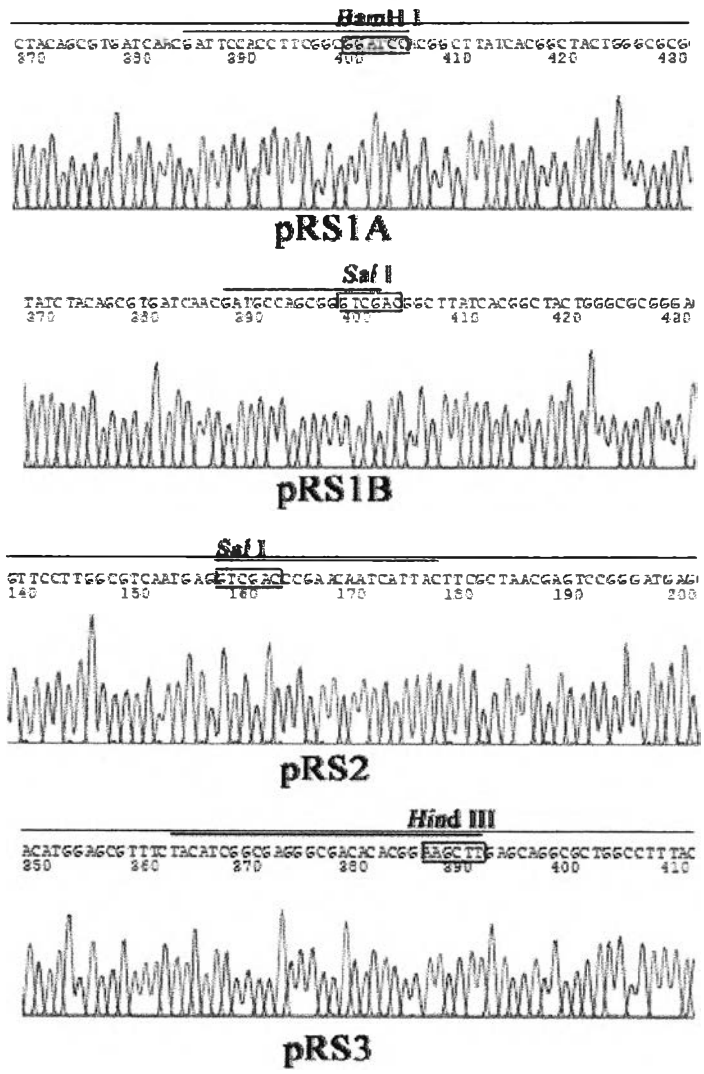


Fig. 3.5. Nucleotide sequencing of mutant regions I_A, I_B, II, and III in pRS1A, 1B, 2 and 3, respectively. The new restriction sites are boxed. Each mutation region is indicated by a line over the region.

3.4. Construction of the mutant CGTases

The DNA fragments containing the mutation regions were subcloned into the original plasmid (pVR328) replacing the wild type sequence to avoid other possible mutation sequences in the mutated plasmids. Then, the resulting plasmids were used for the construction of mutant CGTases that had the combinations of mutation regions.

To subclone the mutation regions into the pVR328, the DNA fragments between the restriction sites indicated in Fig. 3.6 were removed from pRS1A and 1B as the 608 bp *Nsi*I-*Sph*I fragments, and pRS2 and 3 as the 680 bp *Nde*I-*Sac*II fragments, and replaced the corresponding fragments in pVR328. These resulted in pRS4A, 4B, 5 and 6, respectively. The combination of mutant regions was further constructed. The cloning sites were *Nde*I and *Sac*II, which covered the mutation regions II and III. The pRS7A, 7B, 8A, 8B, 9, 10A and 10B were mutant plasmids containing the mutation regions I_A+II, I_B+II, I_A+III, I_B+III, II+III, I_A+II+III and I_B+II+III, respectively (Fig. 3.6).

The plasmid pRS7A, 7B, 8A and 8B were constructed by subcloning the mutation regions II and III in place of the wild type sequences in pRS4A and 4B. Thus, the pRS7A contained additional *Bam*HI and *Sal*I sites and the pRS7B contained two additional *Sal*I sites. The pRS8A and the pRS8B contained the additional *Bam*HI and *Hin*dIII sites and the additional *Sal*I and *Hin*dIII sites, respectively.

The pRS9, which contained regions II and III with the additional *Sal*I and *Hin*dIII sites, was constructed by mutating the single-stranded pRS6 DNA with primer C using USE mutagenesis procedure. This was due to the fact that there were no appropriate restriction sites between regions II and III that could be used for cloning. After obtaining the mutant plasmid pRSCD108 with regions II and III mutated, the combination was subcloned into the pVR328 resulting in pRS9. The pRS10A and 10B, which contained all three mutation regions, were constructed by subcloning mutated regions II and III in pRS9 replacing the corresponding sequences in pRS4A and 4B, respectively. The mutated plasmids, pRS10A and 10B, therefore, contained *Bam*HI, *Sal*I and *Hin*dIII sites, and *Sal*I, *Sal*I and *Hin*dIII sites, respectively.

These recombinant plasmids were subjected to restriction digestion to verify not only the mutation regions but also the cloning sites (Fig. 3.7 and 3.8). The pRS4A was digested with *Bam*HI and *Nsi*I+*Sph*I for the mutation regions and the cloning sites giving 4.3+1.5+0.4 kb and 5.6+0.6 kb DNA fragments, respectively (Fig. 3.7 lanes 1 and 2). The pRS4B was digested with *Sal*I and *Nsi*I+*Sph*I for the mutation regions and the cloning sites giving 4.3+1.9 kb and 5.6+0.6 kb DNA fragments, respectively (Fig. 3.7 lanes 3 and 4). The pRS5, 6, 7A, 7B, 8A, 8B, 9, 10A, and 10B were digested with *Nde*I+*Sac*II for the cloning sites resulting in 5.3+0.9 kb DNA fragments (Fig. 3.7 lanes 6, 8, 10 and 12, and Fig. 3.8 lanes 2, 4, 7, 9 and 12). The pRS5 was digested with *Sal* I for the mutation regions resulting in 4.8+1.4 kb DNA fragments (Fig. 3.7 lane 5). The pRS6 was digested with *Hin*dIII for the mutation regions resulting in 4.4+1.8 kb DNA fragments (Fig. 3.7 lane 7). The pRS7A was digested with *Bam*HI+*Sal*I for the mutation regions resulting in 4.2+1.1+0.5+0.4 kb DNA fragments (Fig. 3.7 lane 9). The pRS7B was digested with *Sal*I for the mutation regions resulting in 4.3+1.4+0.5 kb DNA fragments (Fig. 3.7 lane 11). The pRS8A was digested with *Bam*HI+*Hin*dIII for the mutation regions resulting in 3.2+1.1+0.8+0.7+0.4 kb DNA fragments (Fig. 3.8 lane 1). The pRS8B was digested with *Sal*I+*Hin*dIII for the mutation regions resulting in 3.2+1.2+1.1+0.7 kb DNA fragments (Fig. 3.8 lane 3). The pRS9 was digested with *Sal*I and *Hin*dIII for the mutation regions resulting in 4.8+1.4 kb and 4.4+1.8 kb DNA fragments, respectively (Fig. 3.8 lanes 5 and 6). The pRS10A was digested with *Bam*HI+*Sal*I+*Hin*dIII for the mutation regions resulting in 3.2+1.1+0.8+0.5+0.4+0.2 kb DNA fragments (Fig. 3.8 lane 8). The pRS10B was digested with *Sal*I and *Hin*dIII for the mutation regions resulting in 4.3+1.4+0.5 kb and 4.3+1.9 kb DNA fragments, respectively (Fig. 3.8 lanes 10 and 11).

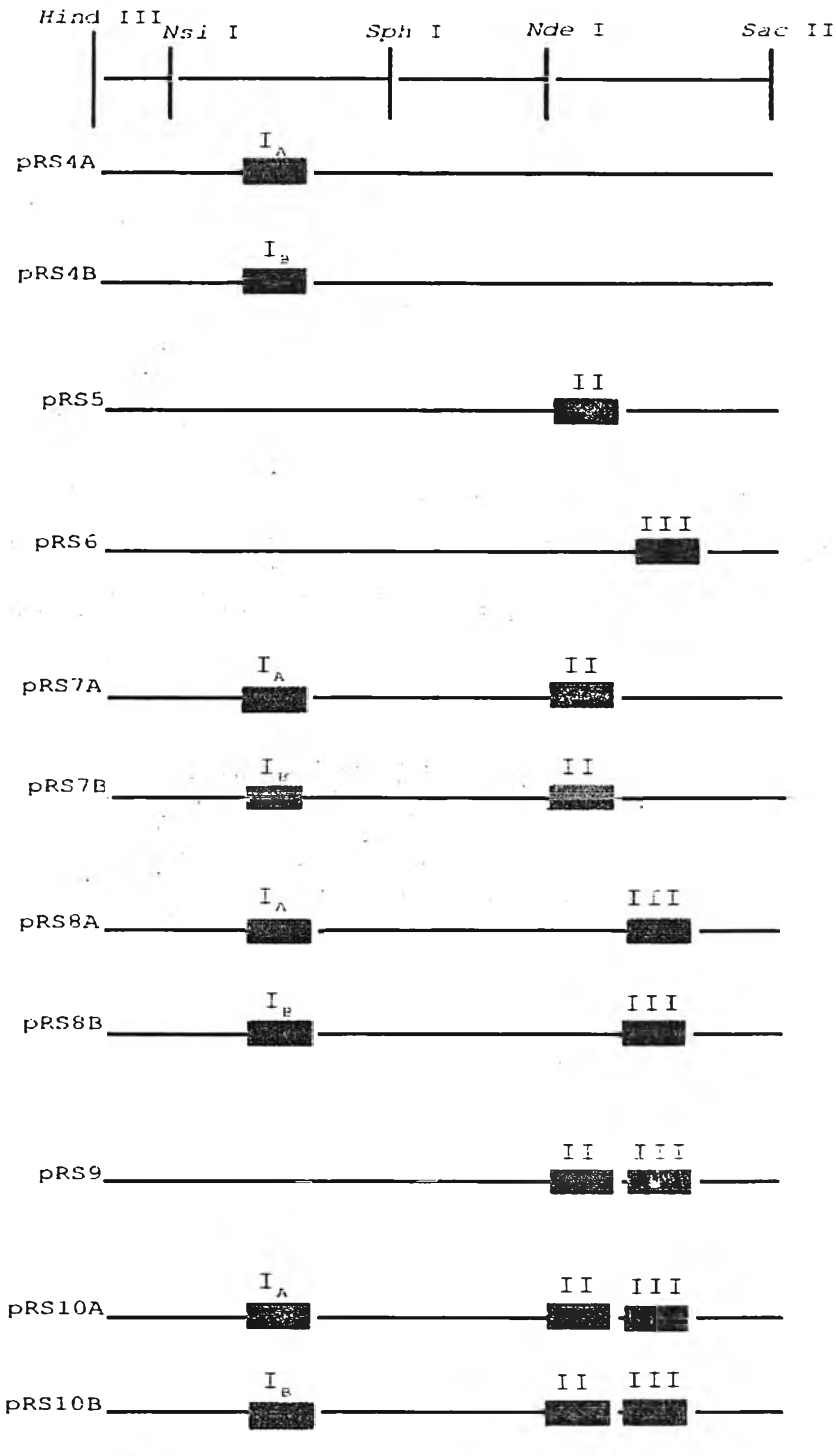


Fig. 3.6. Summary of the mutant CGTase constructs. Regions I_A, I_B, II, and III are the mutated regions containing the restriction sites, *Bam*HI, *Sal*I (for I_B and II) and *Hin*dIII, respectively.

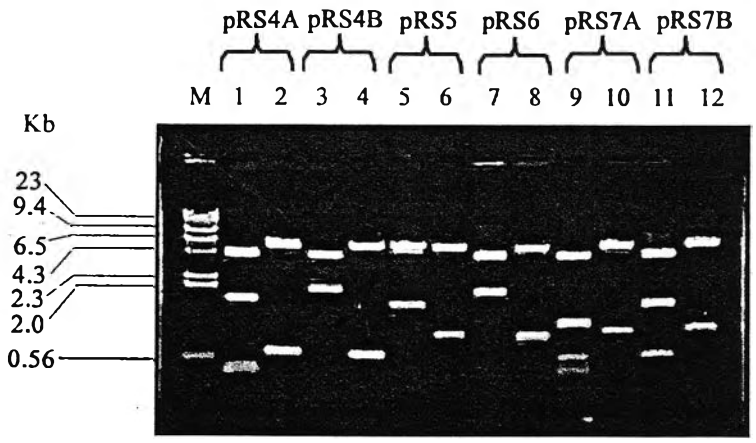


Fig. 3.7. Restriction digestion of pRS4A, 4B, 5, 6, 7A and 7B. Lane M: λ*Hin*dIII marker; lanes 1 and 2: pRS4A digested with *Bam*HI and *Nsi*I+*Sph*I, respectively; lanes 3 and 4: pRS4B digested with *Sal*I and *Nsi*I+*Sph*I, respectively; lane 5 and 6: pRS5 digested with *Sal*I and *Nde*I+*Sac*II, respectively; lane 7 and 8: pRS6 digested with *Hin*dIII and *Nde*I+*Sac*II, respectively; lane 9 and 10: pRS7A digested with *Bam*HI+ *Sal*I and *Nde*I+*Sac*II, respectively; lane 11 and 12: pRS7B digested with *Sal*I and and *Nde*I+*Sac*II, respectively.

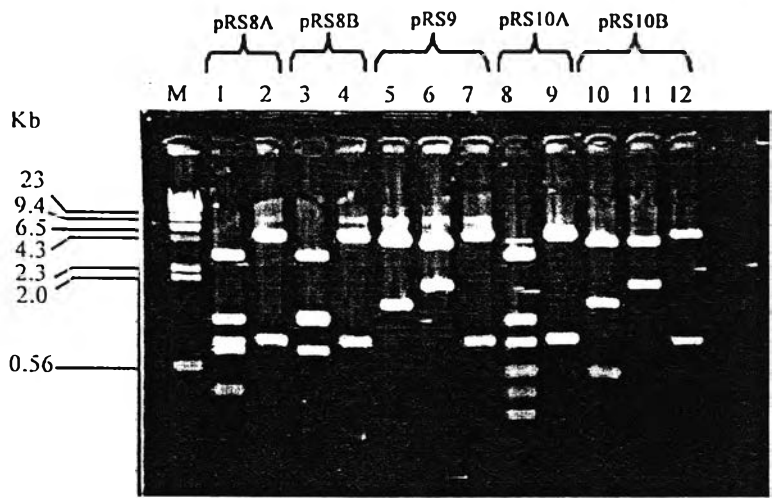
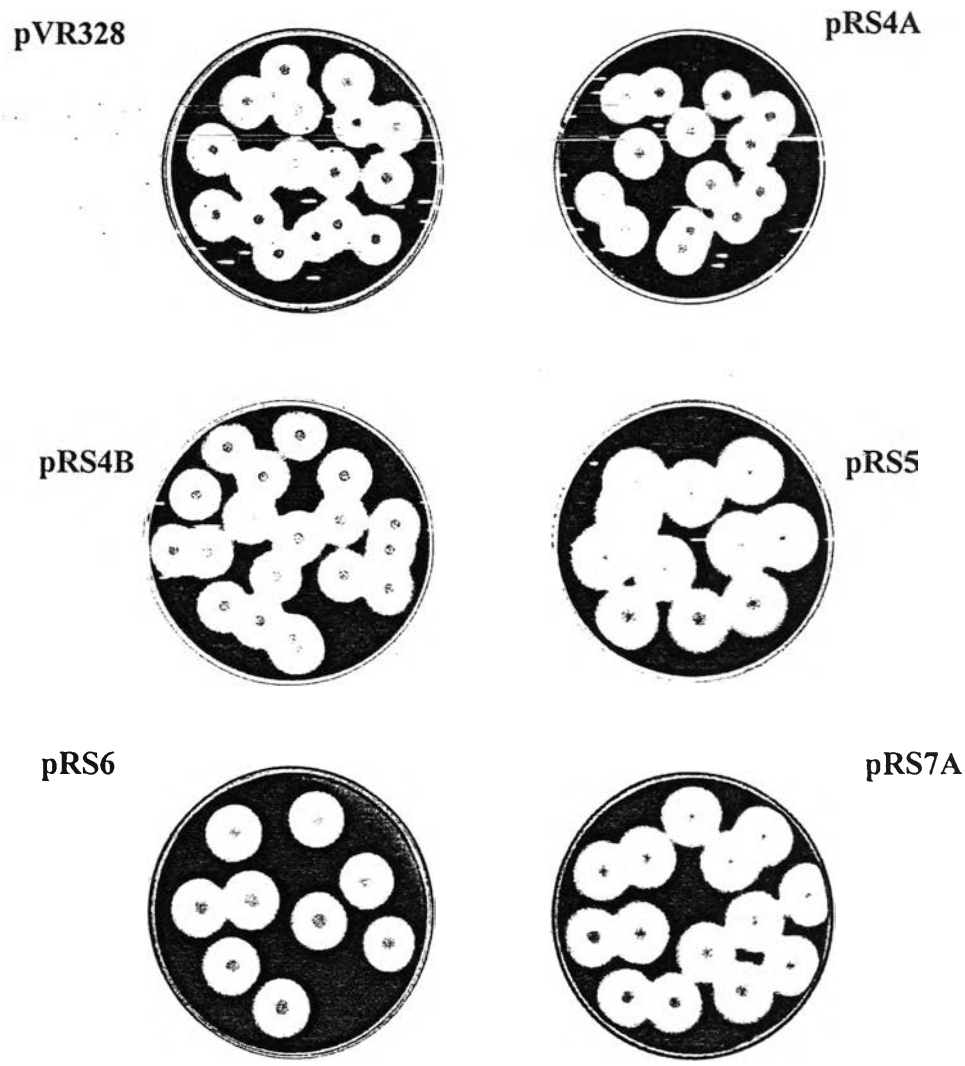


Fig. 3.8. Restriction digestion of pRS8A, 8B, 9, 10A and 10B. Lane M: λ*Hin*dIII marker; lanes 1 and 2: pRS8A digested with *Bam*HI+*Hin*dIII and *Nde*I+*Sac*II, respectively; lanes 3 and 4: pRS8B digested with *Sal*I+*Hin*dIII and *Nde*I+*Sac*II, respectively; lane 5, 6 and 7: pRS9 digested with *Sal*I and *Hin*dIII and *Nde*I+*Sac*II, respectively; lane 8 and 9: pRS10A digested with *Bam*HI+*Sal*I+*Hin*dIII and *Nde*I+*Sac*II, respectively; lane 10, 11 and 12: pRS10B digested with *Sal*I and *Hin*dIII and *Nde*I+*Sac*II, respectively.

3.5. The activities of the mutant CGTases

3.5.1. Halo zone on LB-starch agar

The *E. coli* JM109 transformants harboring each of the mutant CGTase gene was tested for dextrinizing activity on an LB-starch agar plate. Cells with dextrinizing activity gave halo zone surrounding the colonies after exposure to a KI-I_2 indicator solution. Fig. 3.9 shows separately such activity from each mutant clone. Fig. 3.10 summarizes the halo zone assay of all the mutant transformants. All mutant CGTases are dextrinizing active slightly higher than that of wild-type CGTase.



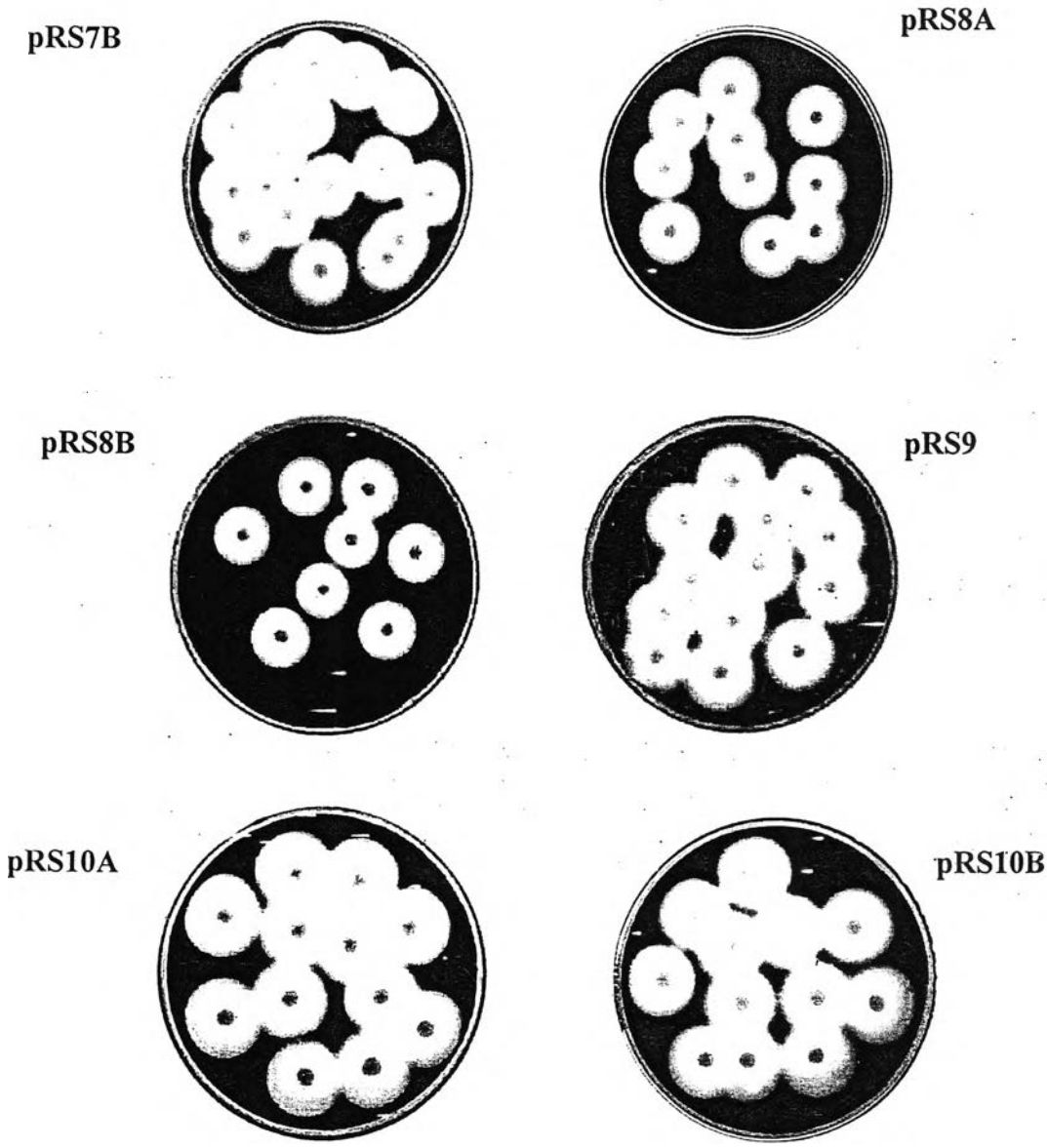


Fig. 3.9. Iodine test for dextrinizing activity of wild type and mutant CGTases. Clear zones surrounding the colonies indicate starch hydrolysis activity.

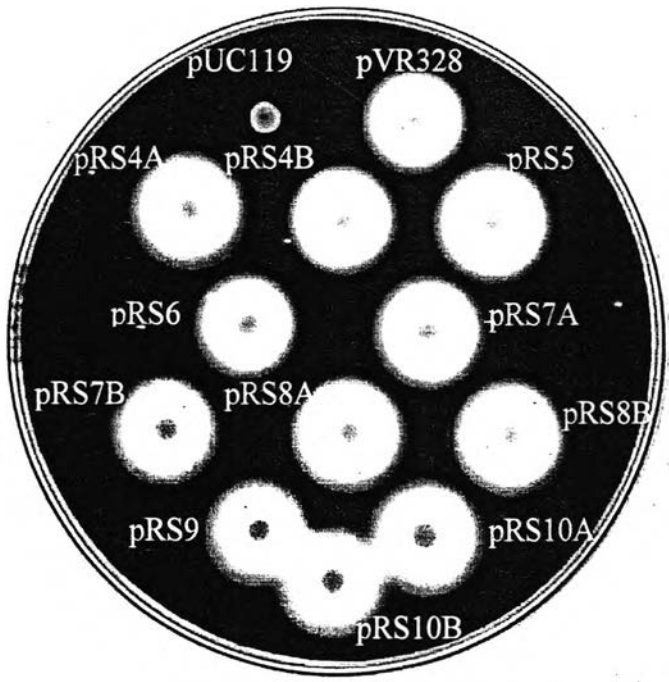


Fig. 3.10. Summary of the iodine test for dextrinizing activity of the wild type and the mutant CGTases. Clear zone surrounding the colonies indicates starch hydrolysis activity.

3.5.2. Dextrinizing activity assay at various temperatures

The crude enzymes in the culture supernatants of the transformants were also assayed for dextrinizing activity using the modified Fuwa method (Fuwa *et al.*, 1954) (Table 3.1). The activities of the wild type and mutant CGTases were measured at different temperatures. The profiles in Fig. 3.11 reveal 3 groups of mutant CGTases with different optimum temperatures. The mutant pRS6 (region III mutant) has more or less the same optimum temperature as the wild type at 65 °C. The pRS4A (I_A), 4B (I_B), 5 (II), 8A (I_A+III), 8B (I_B+III) and 9 (II+III) have lower optimum temperature at about 60 °C. The pRS7A (I_A+II), 7B (I_B+II), 10A (I_A+II+III) and 10B (I_B+II+III) seem to have lower optimum temperature than 60 °C.

Table 3.1. Dextrinizing activity assay of wild type and mutant CGTases at various temperatures.

Dextrinizing activity (unit/mg protein)						
Temperature (°C)	40	50	60	65	70	80
WT	1063.24	2714.54	6082.47	6759.62	6545.78	1437.46
pRS4A	2925.06	4128.89	5747.66	5665.69	3964.96	1275.55
pRS4B	1369.17	2484.70	2959.09	2855.76	2132.42	711.59
pRS5	3440.55	7146.41	9713.99	9405.88	5314.88	522.07
pRS6	1484.60	3015.15	5212.17	5736.14	5396.02	1259.38
pRS7A	1773.04	2741.47	3006.46	2204.25	884.11	19.27
pRS7B	1178.99	1653.32	1692.84	1327.56	760.55	128.12
pRS8A	1243.16	2568.37	4217.70	3778.70	2547.85	717.99
pRS8B	2616.62	4607.92	5840.84	5596.91	4204.34	975.69
pRS9	1120.54	2077.10	2916.89	2810.05	1383.91	360.26
pRS10A	1968.78	3427.56	3478.84	2592.75	1057.04	25.64
pRS10B	1719.08	3134.56	3692.54	2391.95	767.23	155.91

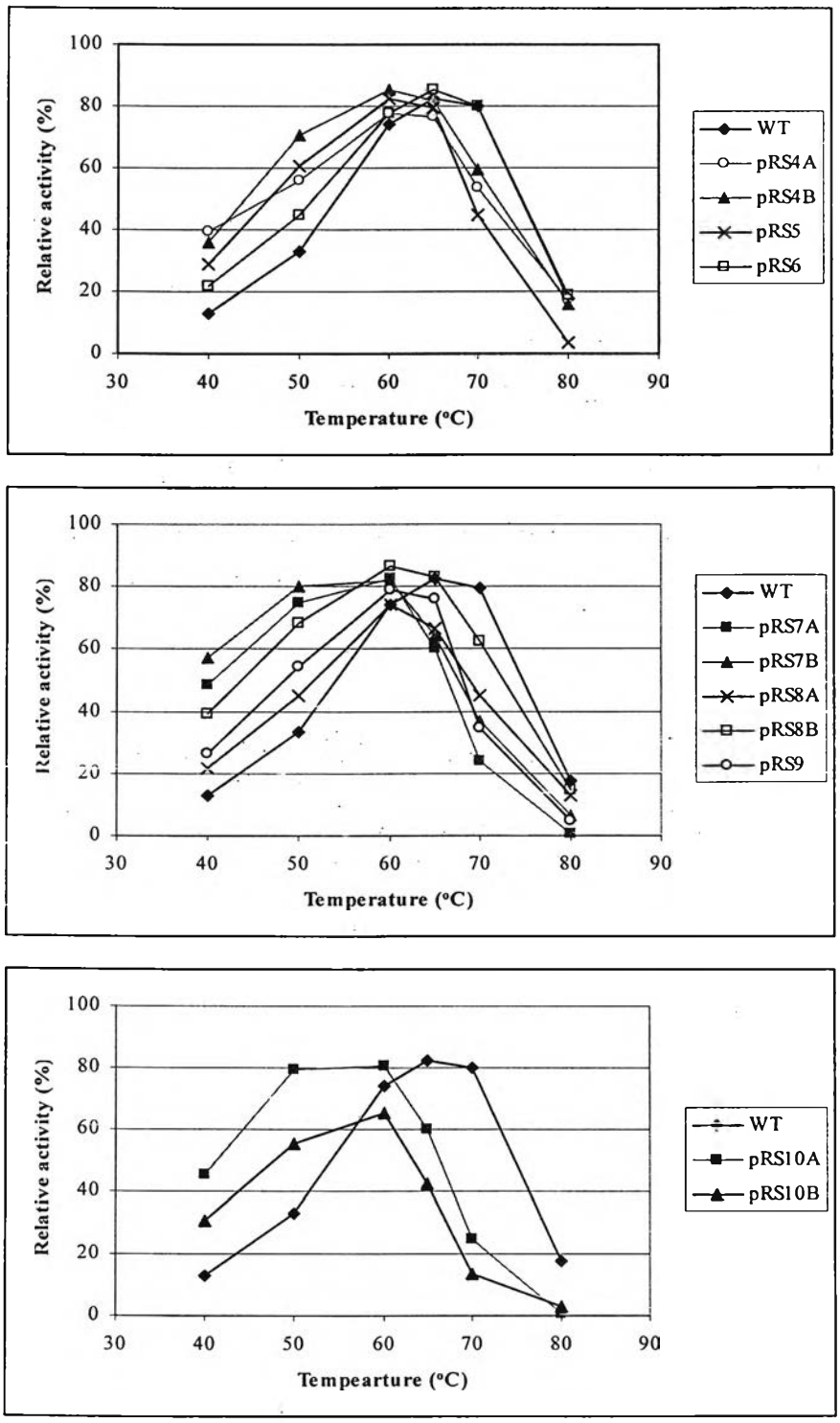


Fig. 3.11. Dextrinizing activity assay of the wild-type and mutant CGTases. The values are shown as relative activity at various temperatures. Each experiment was performed in duplicate.

3.5.3. Thermostability of the CGTases

The effect of temperature on the stability of CGTases was also investigated (Table 3.2). The activities of crude enzymes from the wild type and mutant CGTases were compared at various temperatures, and the percentages of relative activity are shown in Fig. 3.12. In general, the profiles indicate that all mutated CGTases exhibit lower stability than the wild type CGTase except that of the pRS6 mutant, which has comparable stability to the wild type CGTase.

Table 3.2. Specific activities of wild type and mutant CGTases in the thermostability assay.

Thermostability activity (unit/mg protein)							
Temperature (°C)	40	50	55	60	65	70	80
WT	1416.13	1336.54	1183.92	1130.50	187.51	62.14	9.81
pRS4A	2997.89	2916.73	2619.15	1005.85	204.12	174.61	86.08
pRS4B	1475.68	1380.81	1313.37	602.39	171.46	129.17	50.29
pRS5	1965.04	1935.20	1793.83	219.91	190.06	164.93	47.12
pRS6	1348.97	1333.31	1266.20	1173.36	271.81	85.01	49.22
pRS7A	2790.61	2756.64	2465.44	317.89	116.48	75.23	2.43
pRS7B	1477.30	1396.29	1039.33	141.41	123.57	98.85	68.65
pRS8A	3580.52	3052.48	2953.65	1567.18	327.56	268.26	149.66
pRS8B	2841.23	2830.07	2693.81	1819.33	303.78	230.07	122.85
pRS9	1430.59	1385.85	1286.69	355.53	114.88	89.49	9.67
pRS10A	1677.51	1445.04	1179.56	305.65	167.89	74.62	31.57
pRS10B	2388.90	2100.87	1502.24	367.09	169.43	60.24	30.12

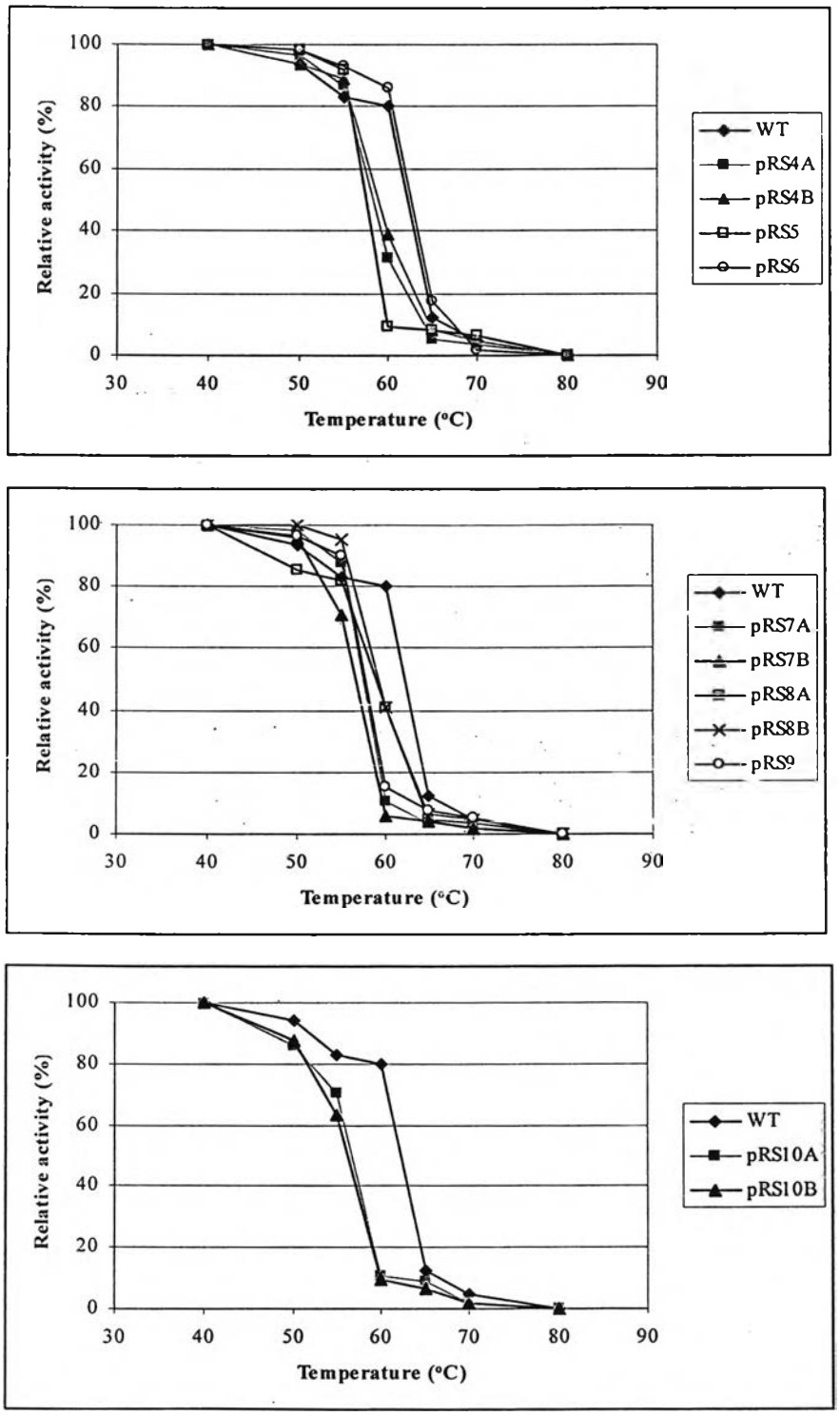


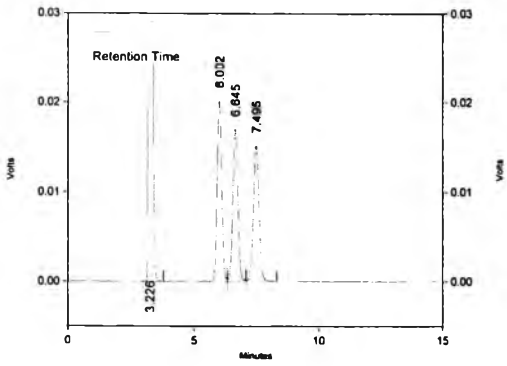
Fig. 3.12. Thermostability of the wild type and mutant CGTases. The values are shown as relative activity at various temperatures. Each experiment was performed in duplicate.

3.5.4. Cyclodextrin forming activity

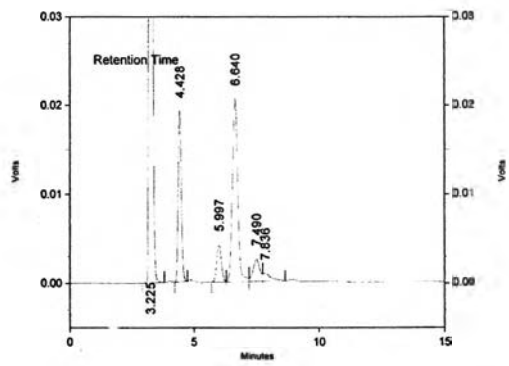
Cyclodextrin production by the mutant CGTases was determined by using HPLC. The crude enzyme from the culture of each mutant plasmid was incubated with soluble starch at 37 ºC for 12 hours. The reaction was terminated by boiling the mixture for 10 minutes. Then, 6U of glucoamylase were added, and the mixture was incubated for 4 hours to convert the linear oligosaccharides to glucose. All mutant enzymes exhibited the cyclodextrin forming activity.

The results were shown in Fig. 3.13. All the mutant enzymes produced β-cyclodextrin as a major product and, hence, were β-CGTases. The pRS4A, 4B, 7A, 7B, 10A and 10B had increased proportion of α-cyclodextrin production, while that of β-cyclodextrin production was decreased significantly and the γ-cyclodextrin production remained unchanged. The pRS5 had proportion of α-, β-, γ- cyclodextrin production similar to that of the wild type. The pRS6 had proportion of α-, and β-cyclodextrin production while that of γ-cyclodextrin production were decreased significantly. The pRS8A and 8B had increased proportion of α-cyclodextrin production and produced slightly lower both proportion of β- and γ-cyclodextrins. The pRS9 had increased proportion of β-cyclodextrin production while the α- and γ-cyclodextrin production were decreased.

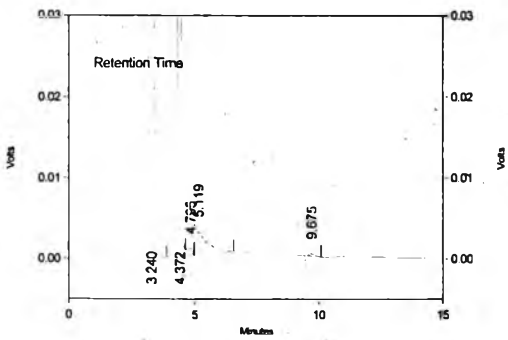
The ratio of cyclodextrins produced is an important characteristic of CGTases from various organisms. It was observed from the HPLC elution profiles that some of the mutant CGTases had altered product specificity. To measure the ratios, the peak area of each cyclodextrin in the HPLC profiles was determined. The cyclodextrin ratios were then calculated as percentage of the total peak areas of α-, β- and γ-cyclodextrins. Table 3.3 summarizes the two determinations of the CD-forming activity.



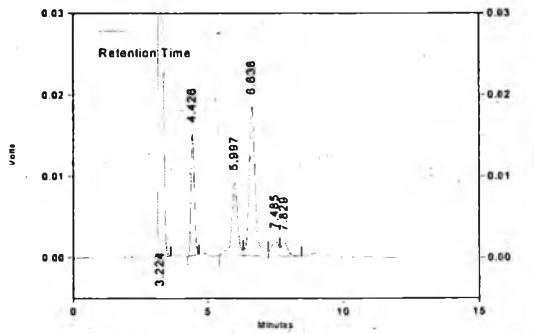
Standard α-,β- and γ-cyclodextrins



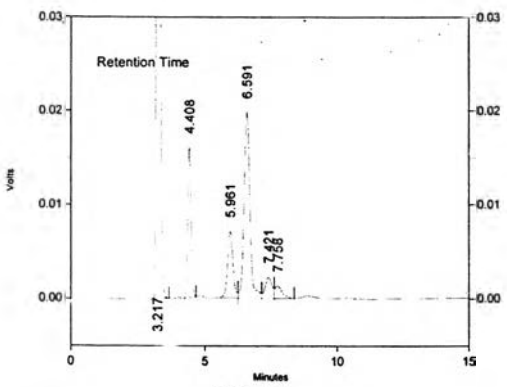
wild type



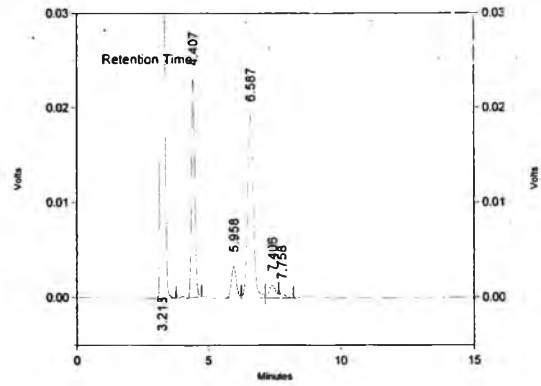
pUC119 (negative control)



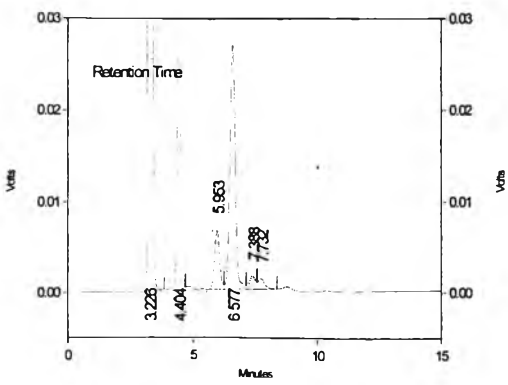
pRS4A



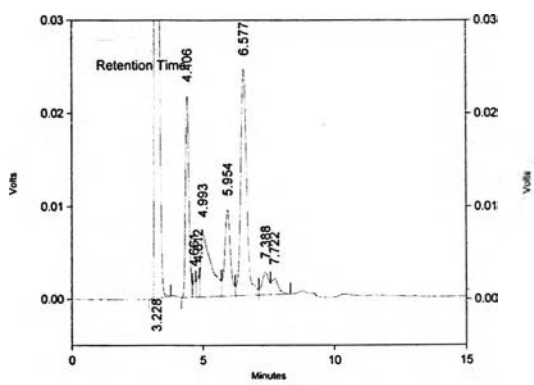
pRS4B



pRS5



pRS6



pRS7A

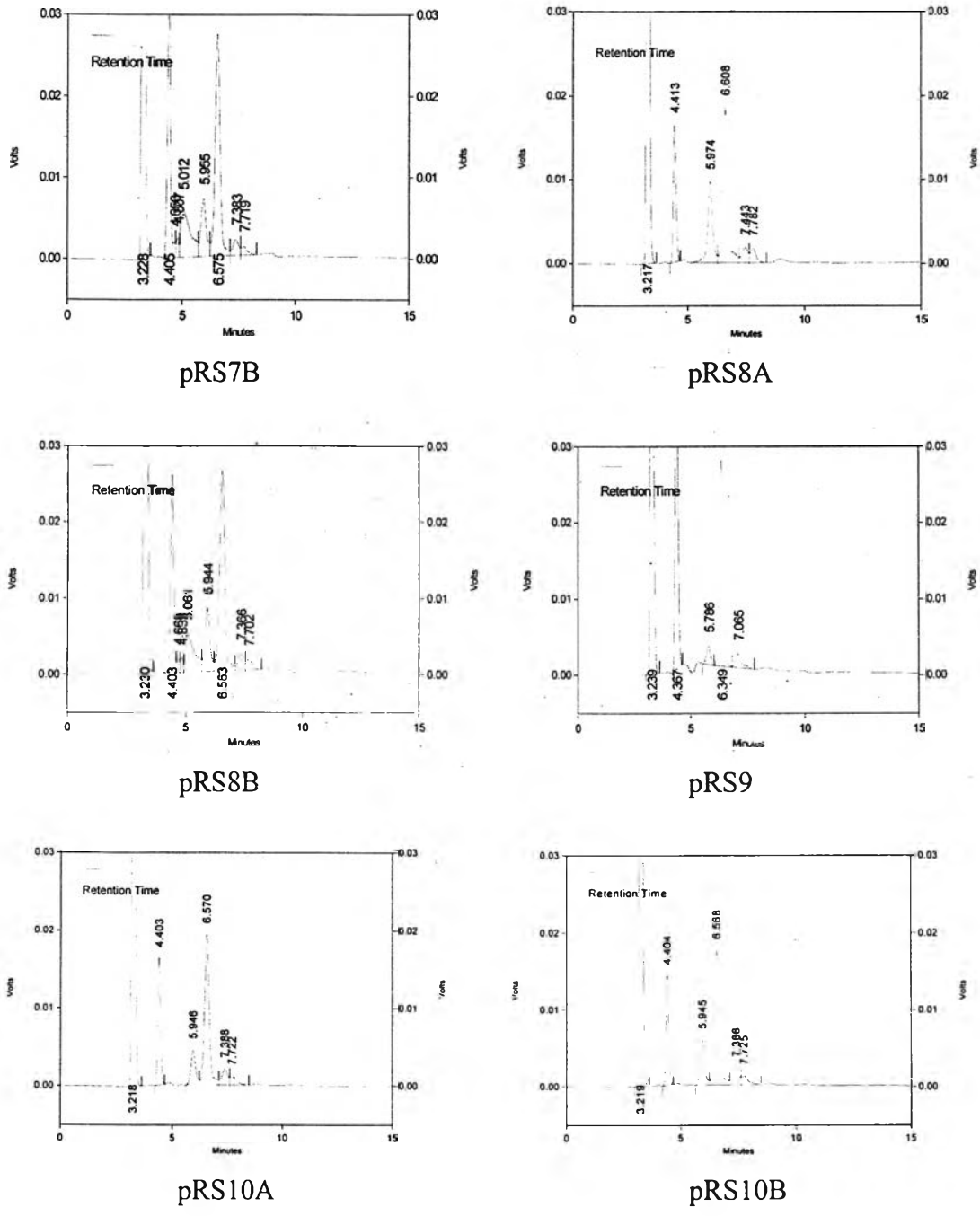
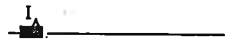
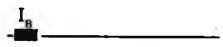
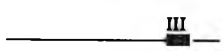
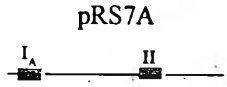
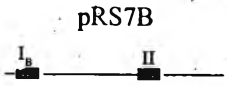
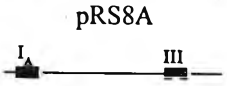
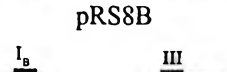
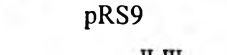
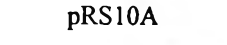
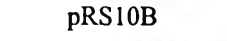


Fig. 3.13. HPLC profiles of cyclodextrins formed by the wild type and mutant CGTases.

Table 3.3. CD-forming activity of the CGTases.

Plasmids	Cyclodextrin ratios, α:β:γ (%)	
	Experiment I	Experiment II
Wild type	12:79:9	13:75:12
pRS4A 	30:63:9	20:73:7
pRS4B 	23:68:9	28:64:8
pRS5 	12:81:7	17:77:6
pRS6 	18:77:5	18:76:6
pRS7A 	25:67:8	27:65:8
pRS7B 	20:74:6	24:66:10
pRS8A 	19:75:6	31:62:7
pRS8B 	23:71:6	20:71:9
pRS9 	7:85:8	9:84:7
pRS10A 	16:74:10	22:68:10
pRS10B 	21:69:10	25:68:7