CHAPTER III

RESULTS

1. Amino acid sequence comparison among various CGTases and the design of mutagenic primers

To determine the differences among the three types of CGTases, the amino acid sequences of the three representative CGTases, α-CGTase from *Bacillus mercerans* , the ratio of α-, β- and γ-CD production is 65:24:11,(Takano *et al.*,1986), β/γ-CGTase from *B. firmus* 290-3, the approximate ratio of β- and γ-CD production is 50:50, (Englbrecht *et al.*, 1990) and γ-CGTase from *B. clarkii* 7364 the ratio of α-, β- and γ-CD production is 7:14:79, (Takada *et al.*, 2003) were downloaded from the GenBank and compared with that of the CGTase from *B. circulans* A11. The amino acid sequence alignment was carried out using clustalX (Thompson *et al.*, 1999) and the result is shown in Fig. 19. The result shows moderate homology(~70%) among the four CGTases as several homologous segments scatter all over the entire amino acid sequences. There are however three major regions in the α- and β-CGTases that are different from those of the β/γ- and γ-CGTases. The three regions (I, II and III) consist of amino acid residues 87-95, amino acid residues 141-152 and amino acid residues 532-536 (*B. circulans* A11 CGTase numbering), respectively. The first and second regions reside in the catalytic domain A/B while the third region, amino acid residues 532-536, is located in domain D.

From the result of this alignment, three primers (1, 2 and 3) were designed corresponding to the three amino acid regions in β-CGTase that are different from those of β/γ- and γ-CGTases. Upon mutagenesis, each primer introduced both base substitutions and deletions (Fig. 20). Amino acid substitutions were made in favor of the amino acid sequence in γ-CGTase. The nucleotide sequences deleted in β-CGTase were 5'-TACTCCGGC-3' (coding for YSG), 5'-TCGGATGATCCTTCCTTT-3' (coding for SDDPSF) and 5'-ACGGCAGTC-3' (coding for TAV) in regions I, II and III, respectively. Moreover, primers 1, 2 and 3 also created *Sma*I, *Eco*RV and *Nru*I restriction sites, respectively, in the mutant plasmids.

It should be noted that there was one additional base substitution in region I as the base was mistakenly changed from the wild type C to T.at amino acid residue 98 as shown in Fig.20. This substitution, however, had no effect on the amino acid sequence; the amino acid remained unchanged.

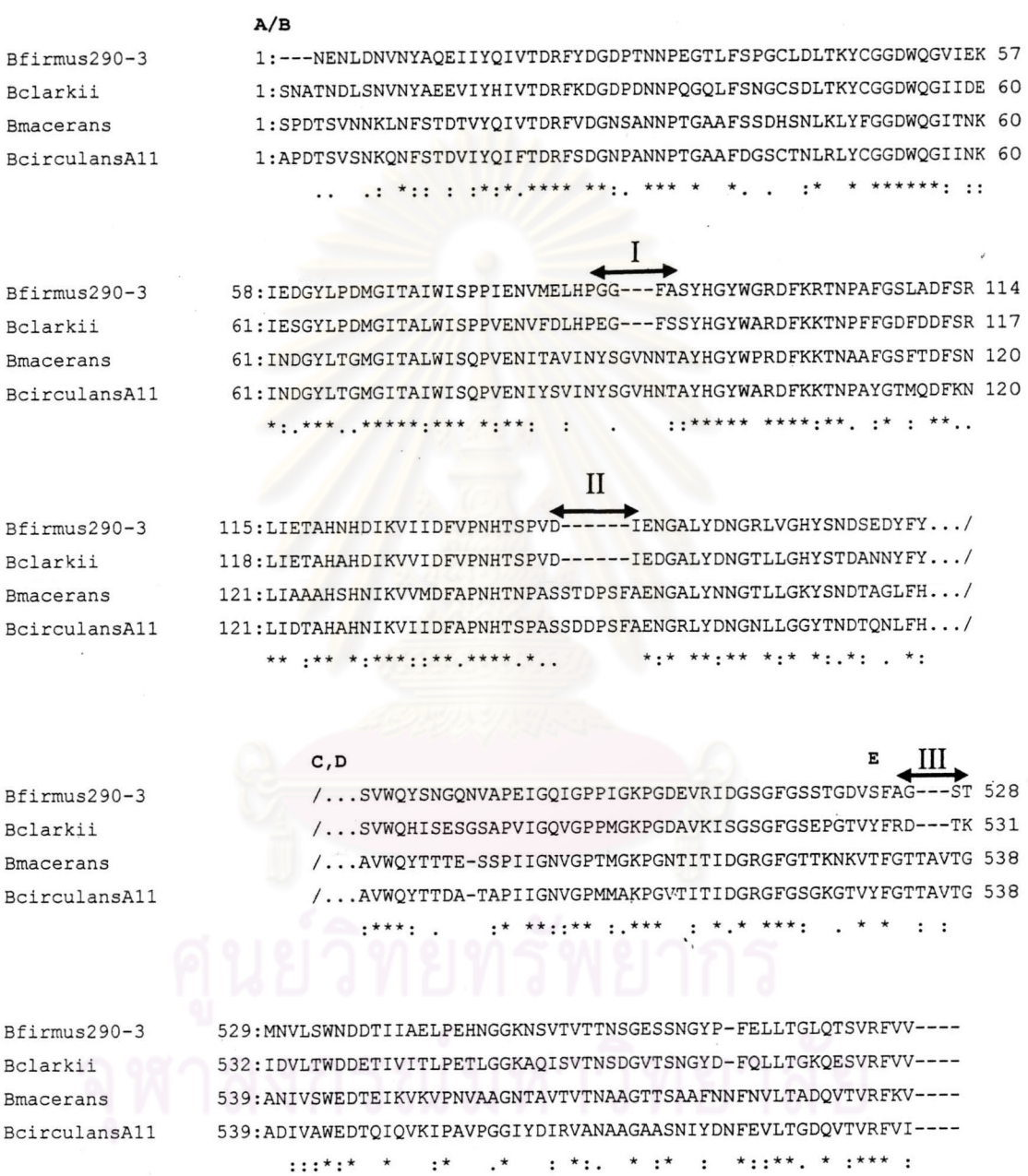


Figure 19. Alignment of the amino acid sequences between β-CGTase from *B. circulans* A11 (BcirculansA11) and other typical CGTases, α-CGTase from *P. macerans* IAM1243 (Bmacerans), β/γ-CGTase from *B. firmus* 290-3 (Bfirmus290-3) and γ-CGTase from *B. clarkii* 7364 (Bclarkii). Double arrows indicate the amino acid sequences of β-CGTase that are different from those of γ-CGTase.

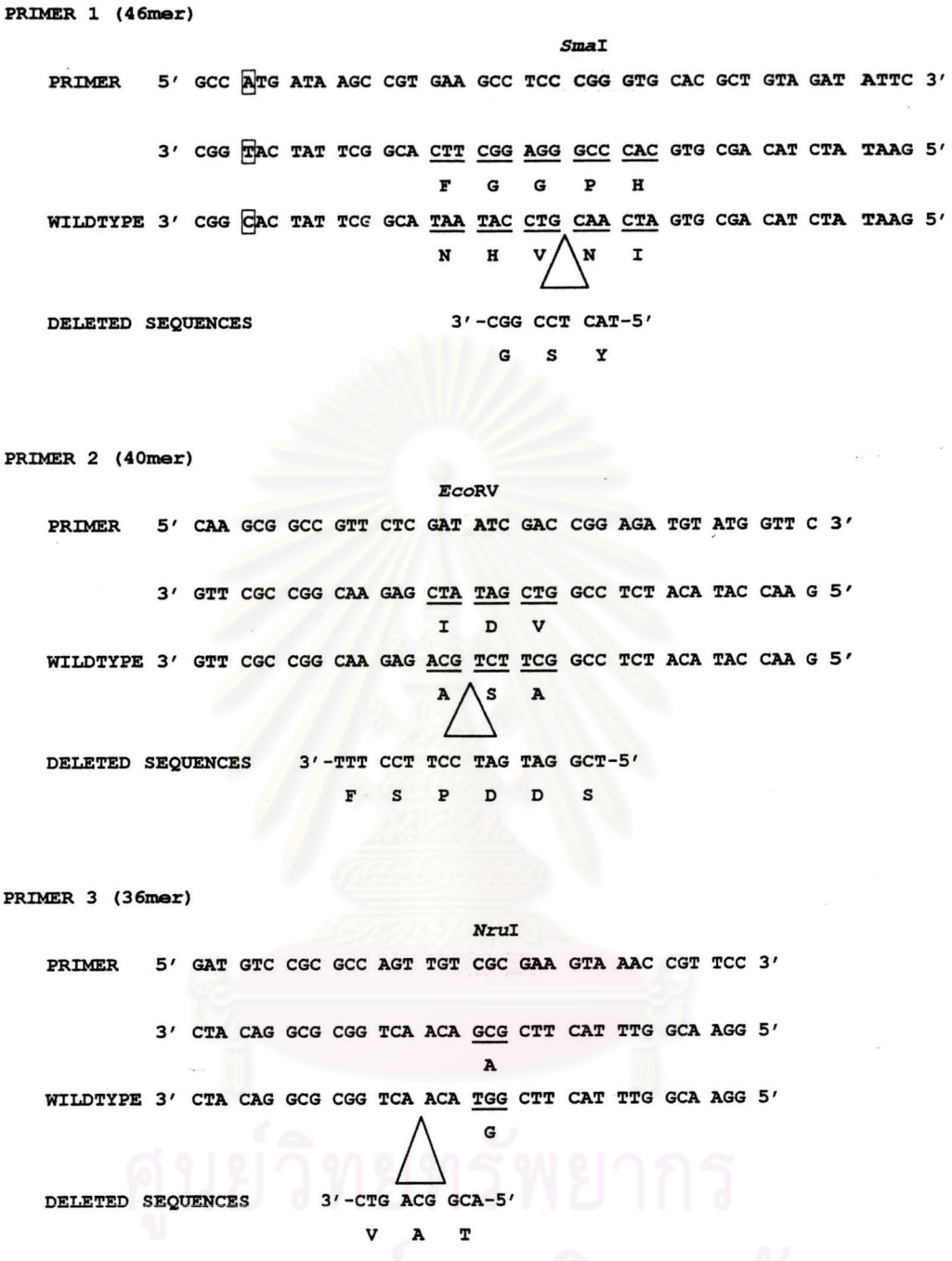


Figure 20. The design of oligonucleotides used in USE procedure and the regions of nucleotide sequences of wild type that used for designing. Newly created restriction recognition sites are shaded. Δs indicate the positions of nucleotides deleted. The base in primer 1 that is mistakenly substituted are boxed.

2. Mutagenesis of β-CGTase gene from *B. circulans* A11 by using USE procedure

The plasmid pVR328, containing the β-CGTase gene from *B. circulans* A11, was used as a starting plasmid for the USE mutagenesis procedure (Fig. 18). The mutagenic primers 1, 2 and 3, along with the reference *Sca*I primer were used to mutate the CGTase genes at 3 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 clones were screened for the presence of restriction sites, *Sma*I, *Eco*RV and *Nru*I, created. The positions of these sites were confirmed by restriction enzyme digestion and agarose gel electrophoresis (Fig. 21). Three mutated plasmids, pNan1, 2 and 3, were obtained as shown in Fig. 22 with the restriction sites, *Sma*I, *Eco*RV and *Nru*I, respectively.

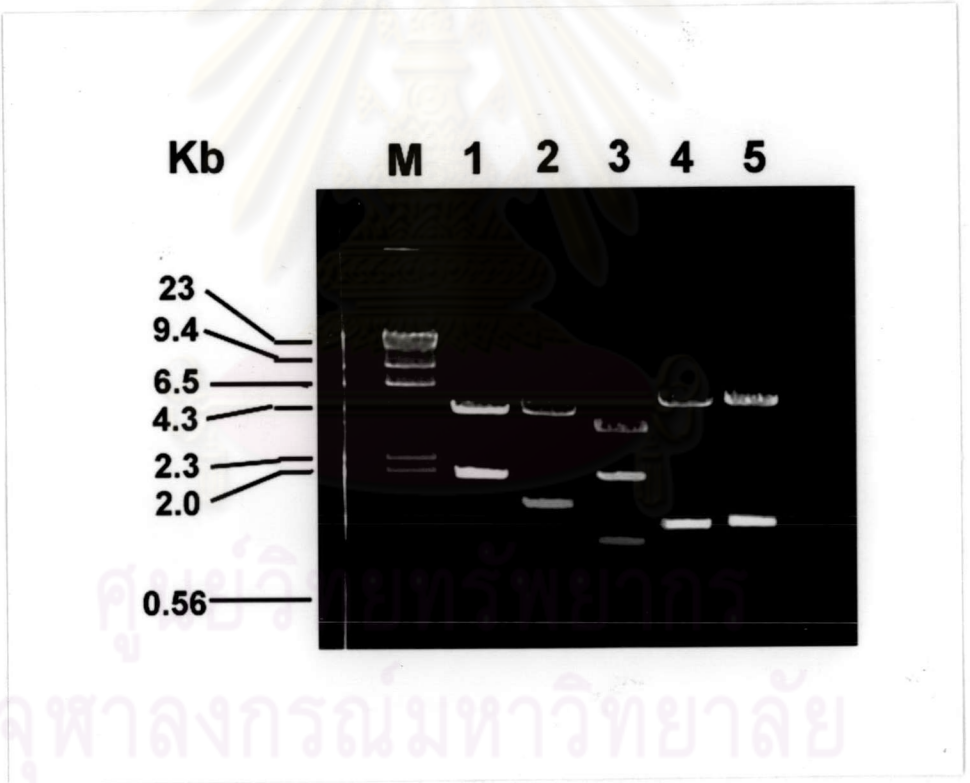


Figure 21. Restriction digestion of pNan1, 2 and 3. Lane M: λ/*Hin*dIII marker; lanes 1, 2 and 3: pNan1 digested with *Sma*I, *Sma*I+*Nde*I and *Sma*I+*Hin*dIII, respectively; lane 4: pNan2 digested with *Eco*RV; lane 5: pNan3 digested with *Nru*I+*Sph*I.

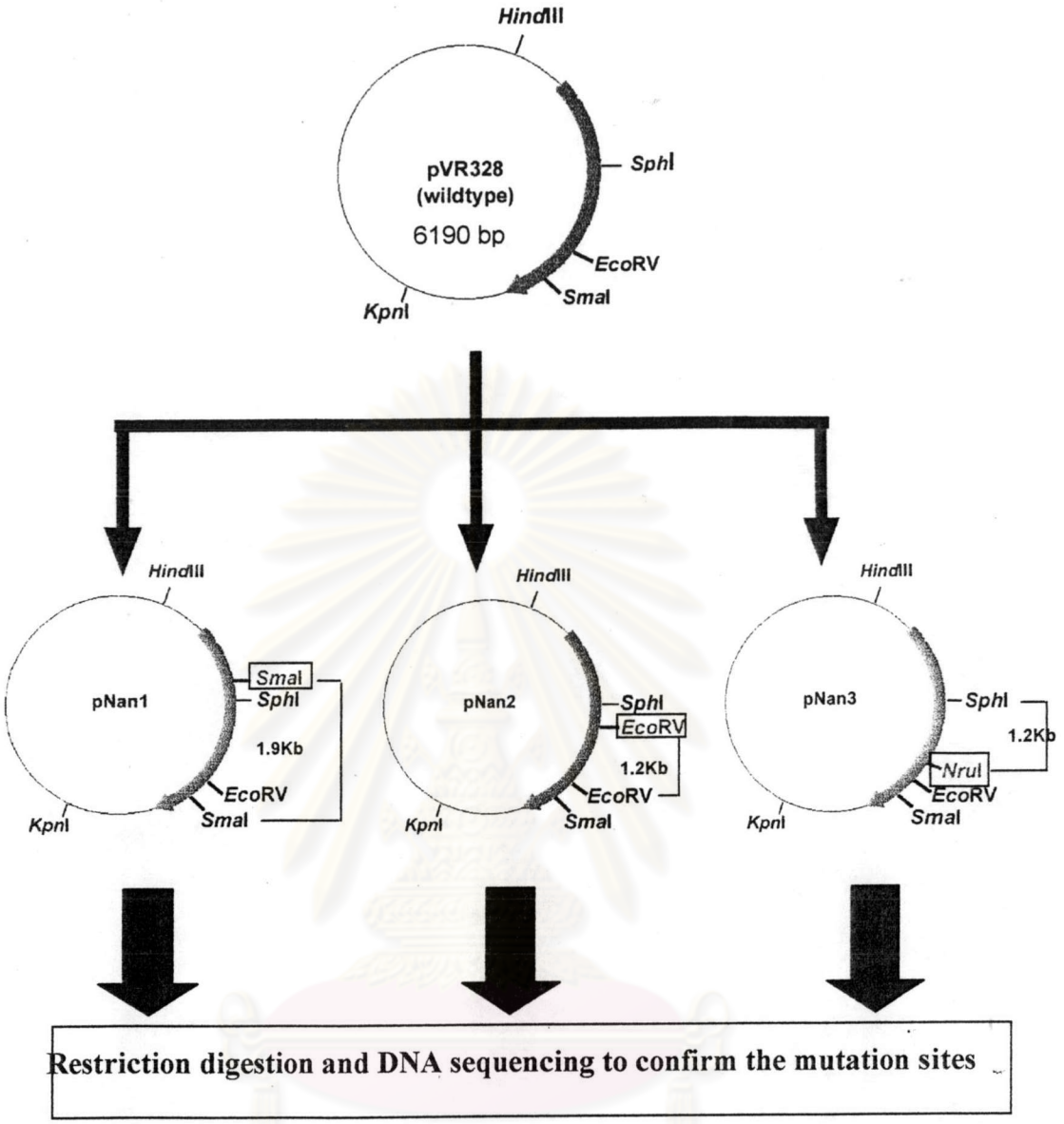


Figure 22. The mutated plasmids (pNan1, 2 and 3). The plasmids were processed to confirm the mutations by restriction digestion and DNA sequencing. The new restriction sites are boxed and the sizes of the DNA fragments analyzed are indicated.

3. DNA sequence determination of the mutation sites

The precise DNA sequences around the new restriction sites in pNan1, pNan2 and pNan3 were sequenced using the dideoxy termination reaction. The three mutants had the nucleotide sequences as designed (Fig. 23). Nucleotide sequences between the two restriction sites border the mutation sites were also checked to make certain that there were no additional mutations. The restriction sites were used subsequently to subclone the mutation sites.

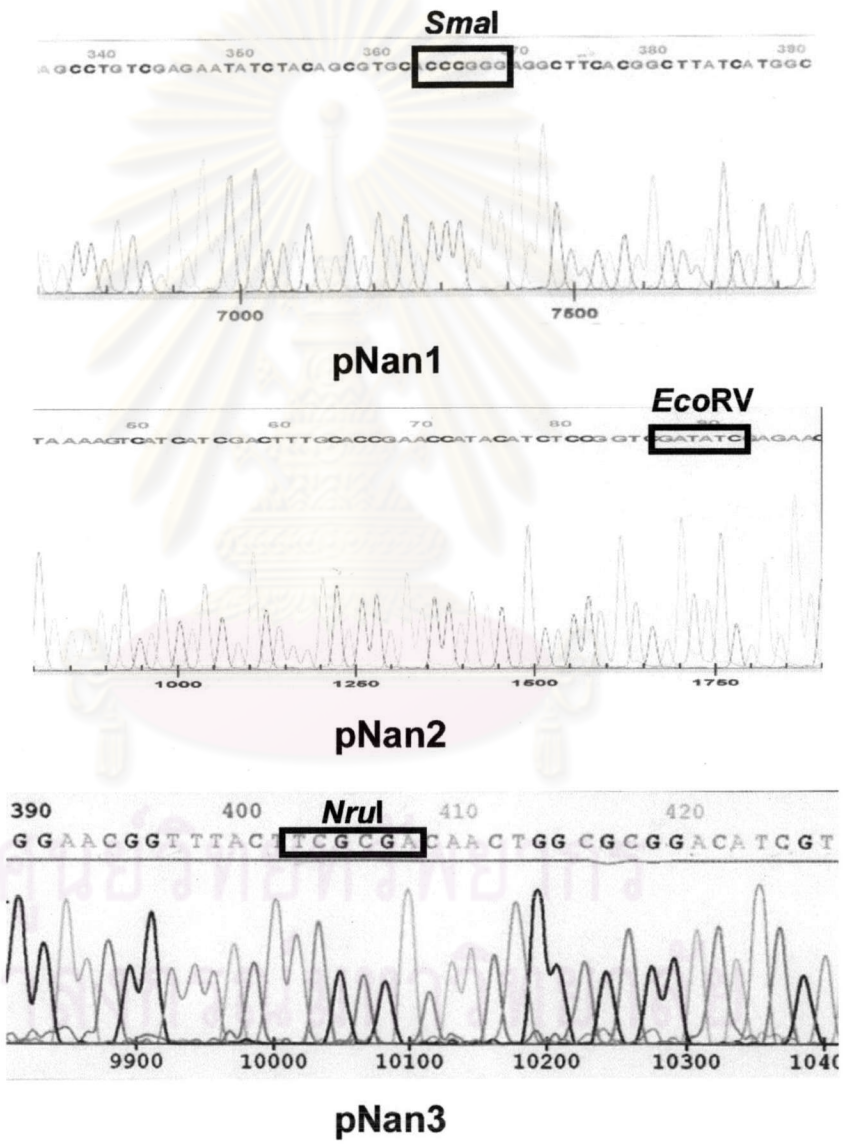


Figure 23. The nucleotide sequences of mutant sites I, II and III in pNan1, 2 and 3, respectively. The new restriction sites are boxed.

4. Construction of the mutant CGTases

The DNA fragments containing the new restriction sites were subcloned into the original plasmid (pVR328) to avoid other possible mutation sequences in the mutated plasmids. The resulting plasmids were used for the construction of mutant CGTases that had the combinations of mutation sites.

To subclone the mutation sites into the pVR328, the DNA fragments between two restriction sites (Fig. 24) were removed from pNan1, 2 and 3 as the 592 bp *Nsi*I-*Sph*I fragment, 293 bp *Sph*I-*Nde*I fragment and 623 bp *Sac*II-*Kpn*I fragment, replacing the corresponding fragments in pVR328. These resulted in pNan4, 5 and 6, respectively. The pNan7 and 8 were constructed by subcloning the *Nsi*I-*Sph*I fragment from pNan1 in place of the corresponding fragment in pNan5 and 6, respectively. The pNan9 and 10 were constructed by subcloning the *Sac*II-*Kpn*I from pNan3 in place of the corresponding fragment in pNan5 and 7, respectively.

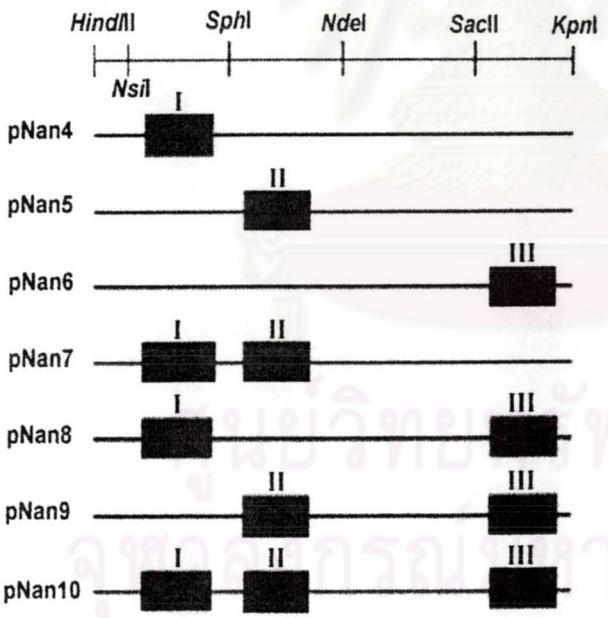


Figure 24. Summary of the mutant CGTase conctructs. Regions I, II and III are the regions that mutated and contains the restriction sites, *Sma*I, *Eco*RV and *Nru*I, respectively.

Subcloning of mutation sites I, II and III in place of the wild type sequences in pVR328 gave rise to pNan4, 5 and 6 which, in fact, were almost identical to pNan1, 2 and 3, respectively, with the exeption of site. The pNan4 then had the new *Sma*I site, pNan5 had the new *Eco*RV site and pNan6 had the new *Nru*I site. These restriction sites were used for the screening of the subclones (Fig. 25, lanes 1, 3 and 5). The cloning sites were also checked (Fig. 25, lanes 2, 4 and 6).

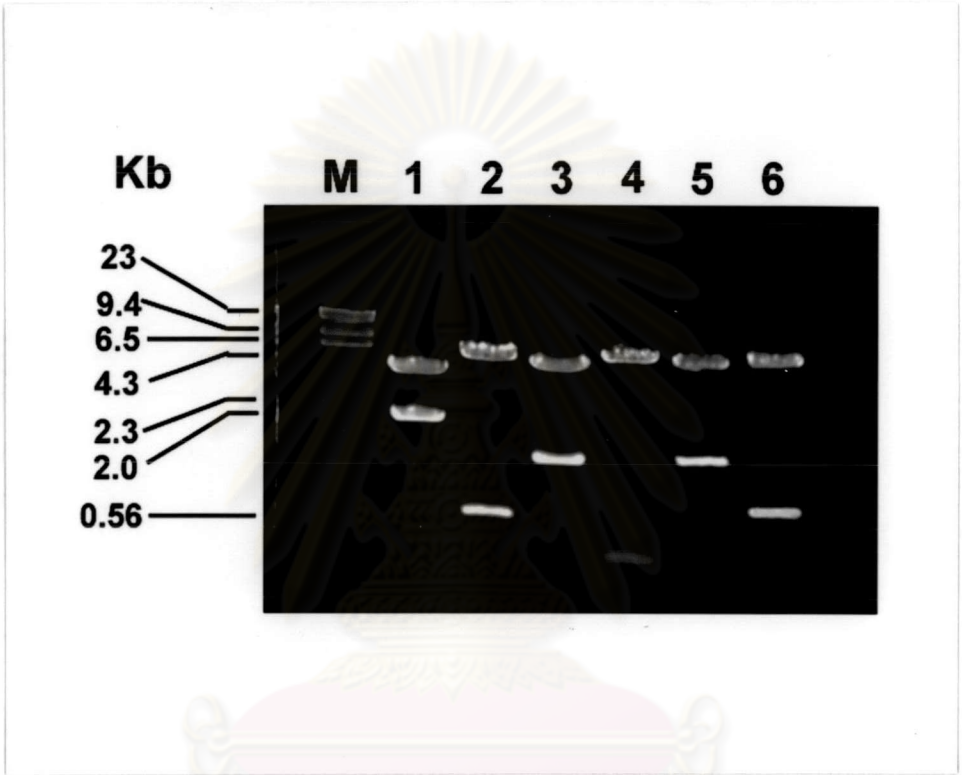


Figure 25. Restriction digestion of pNan4, 5 and 6. Lane M: λ/*Hin*dIII marker; lanes 1 and 2: pNan4 digested with *Sma*I and *Nsi*I+*Sph*I, respectively; lanes 3 and 4: pNan5 digested with *Eco*RV and *Sph*I+*Nde*I, respectively; lane 5 and 6: pNan6 digested with *Nru*I+*Sph*I and *Sac*II+*Kpn*I, respectively.

Plasmid pNan7 was constructed to contain the mutation sites I and II by subcloning mutation site I in place of the wild type sequences in pNan5. The pNan7 should contain the additionanl *Sma*I and *Eco*RV sites that were confirmed by restriction digestion (Fig. 26, lane 1 and 2). The cloning site, *Nsi*I-*Sph*I, was also checked (Fig. 26, lane 3). Plasmid pNan8 was constructed to contain the mutation sites I and III by subcloning mutation site I in place of the wild type sequences in pNan6. The pNan8 should contain the new *Sma*I and *Nru*I sites that were confirmed by restriction digestion (Fig. 26, lane 4 and 5). The cloning site, *Nsi*I-*Sph*I, was also checked (Fig. 26, lane 6). Plasmid pNan9 was constructed to contain the mutation sites II and III by subcloning mutation site III in place of the wild type sequences in pNan5. The pNan9 should contain the new *Eco*RVand *Nru*I sites that were confirmed by restriction digestion (Fig. 26, lane 7 and 8). The cloning site, *Sac*II-*Kpn*I, was also checked (Fig. 26, lane 9). The last recombinant plasmid in this series was pNan10, which had all three mutation sites, I, II and III, was constructed by subcloning mutation site III in place of the wild type sequences in pNan7. The resulting plasmid should contain the additional *Sma*I, *Eco*RV and *Nru*I sites that were confirmed by restriction digestion (Fig. 27, lane 1, 2 and 3). The cloning site, *Sac*II-*Kpn*I, was also checked (Fig.27 lane 4).

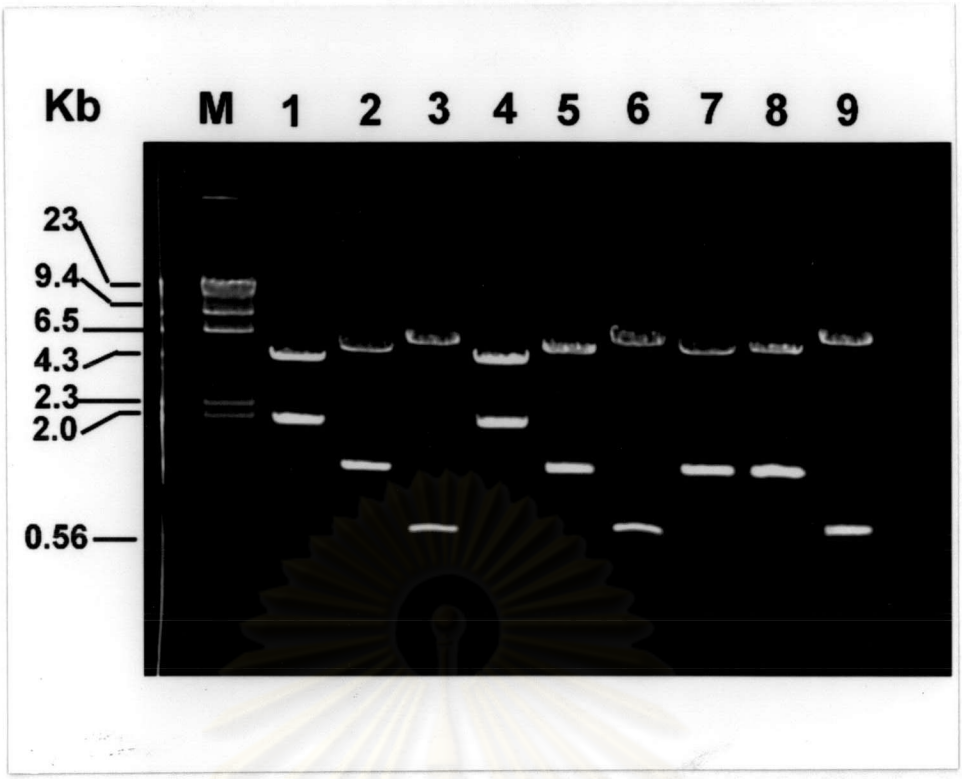


Figure 26. Restriction digestion of pNan7, 8 and 9. Lane M: λ/*Hin*dIII marker; lanes 1, 2 and 3: pNan 7 digested with *Sma*I, *Eco*RV and *Nsi*I+*Sph*I, respectively; lane 4, 5 and 6: pNan8 digested with *Sma*I, *Nru*I+*Sph*I and *Nsi*I+*Sph*I, respectively; lane 7, 8 and 9: pNan9 digested with *Eco*RV, *Nru*I+*Sph*I and *Sac*II+*Kpn*I, respectively.

Figure 27. Restriction digestion of pNan10. Lane M: λ/*Hin*dIII marker; lanes 1: digested with *Sma*I, lane 2: digested with *Eco*RV, lane3: digested with *Nsi*I+*Sph*I, and lane 4: digested with *Sac*II+*Kpn*I.

5. The activities of the mutant CGTases

E. coli JM109 transformant containing each of the mutant CGTase was tested for dextrinizing activity on the LB-starch agar plate. Cells with dextrinizing activity gave halo zone surrounding the colony upon KI-I_2 treatment. Figure 28 shows such activity. Figure 29 summarizes the halo zone assay of all the transformants. The crude enzymes in the culture supernatants of the transformants were also assayed for dextrinizing activity using the Fuwa method (Fuwa *et al.*,1954) (Table 3). The pNan7 had increased dextrinizing activity, while the others had slightly lower dextrinizing activity.

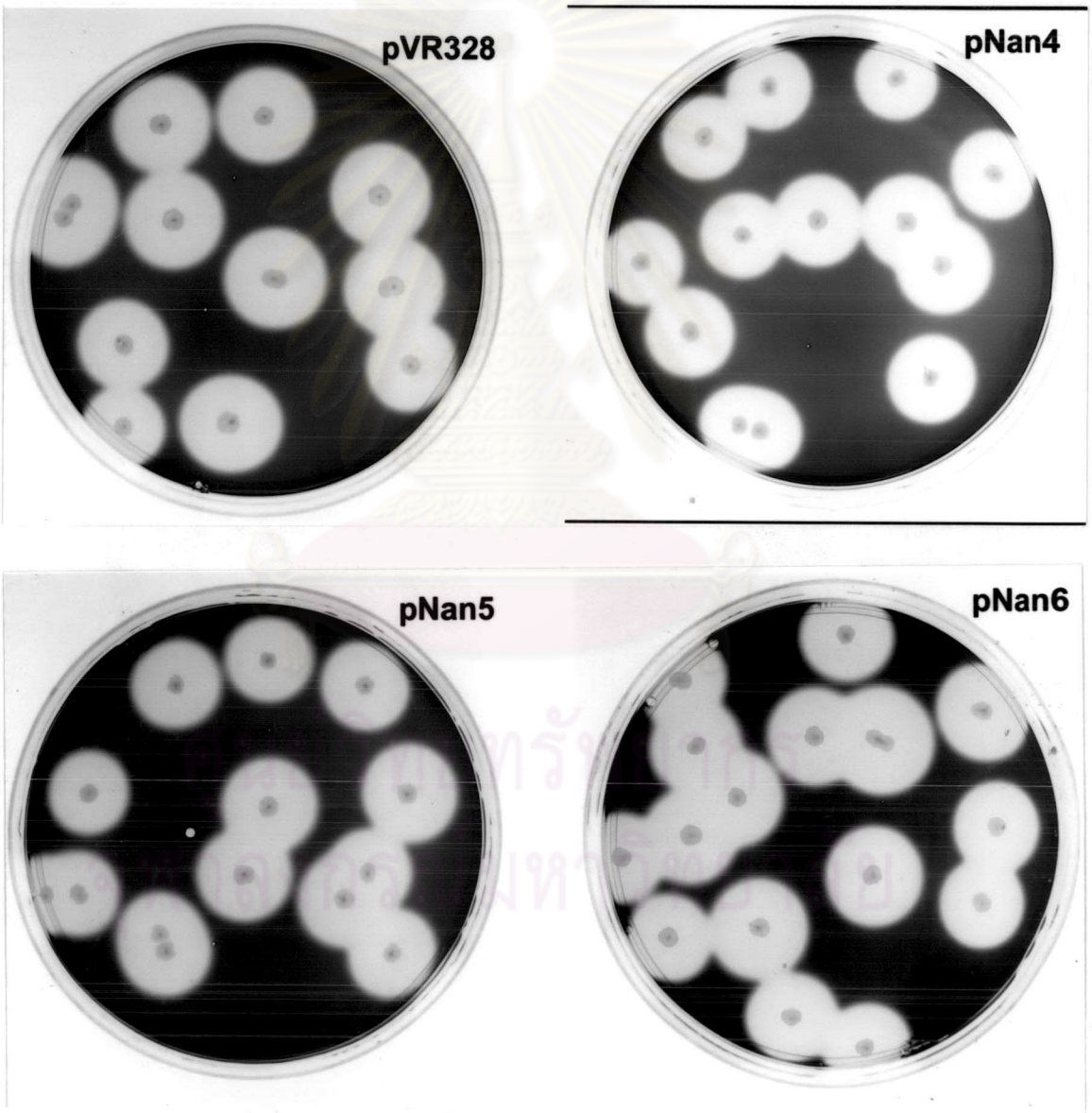


Figure 28. Iodine test for dextrinizing activity of wild type and mutant CGTases. Clear zones surrounding the colonies indicate starch hydrolysis activity.

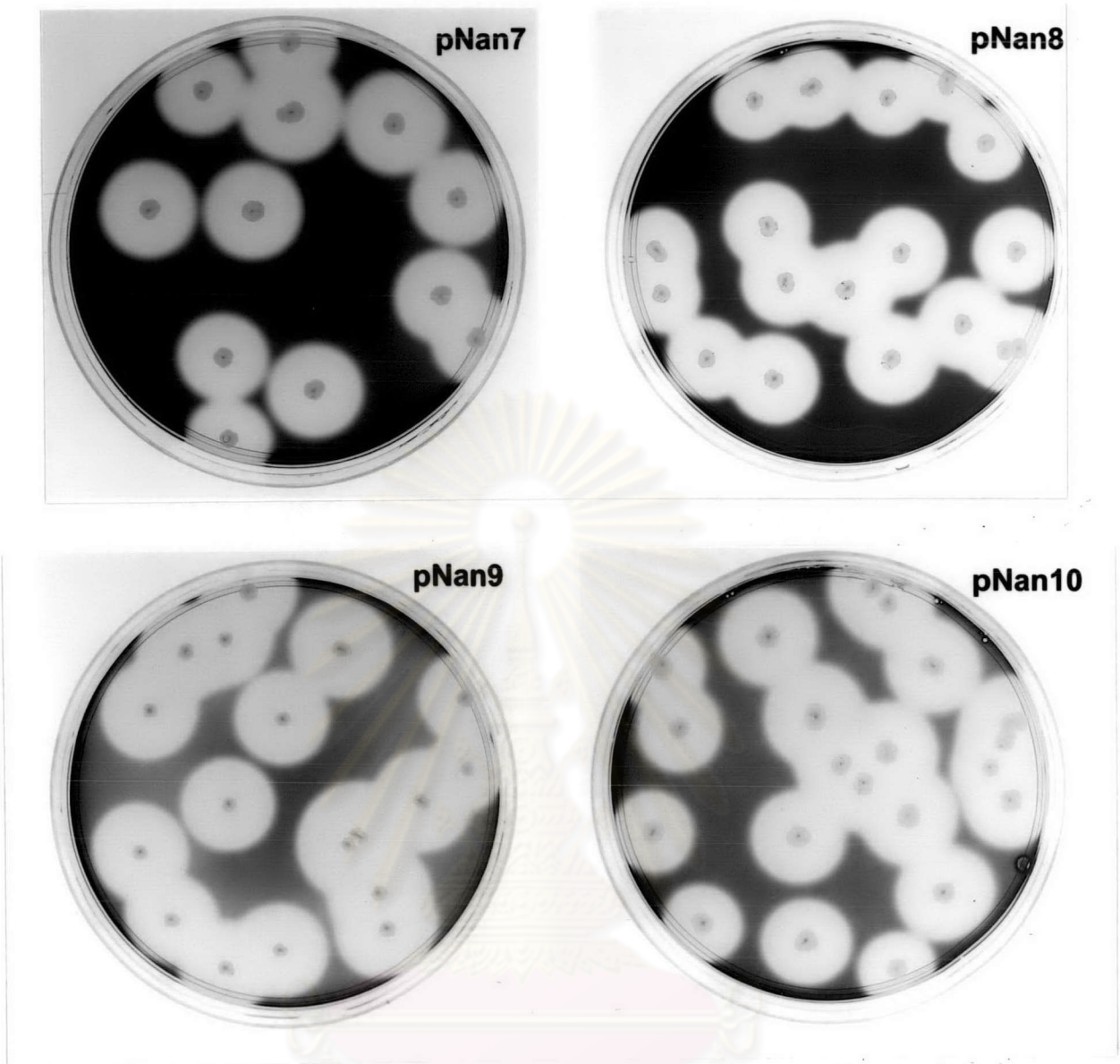


Figure 28. (continue) Iodine test for dextrinizing activity of the wild type and the mutant CGTases. Clear zones surrounding the colonies indicate starch hydrolysis activity.

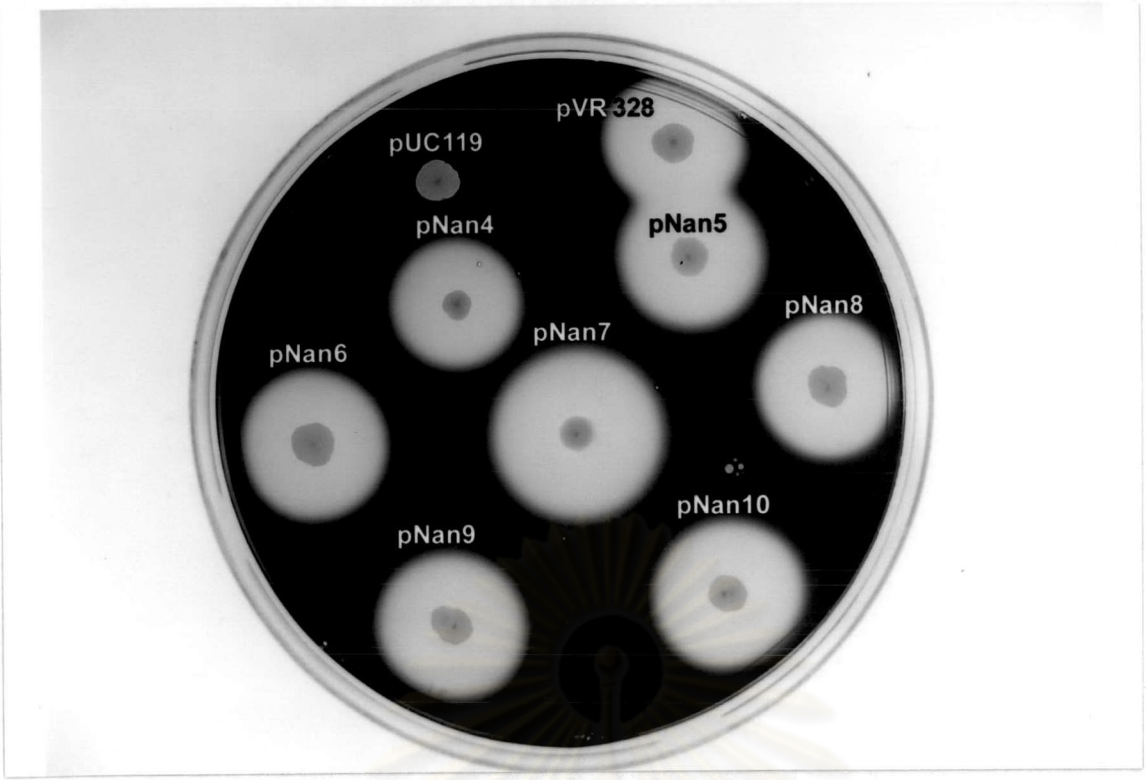
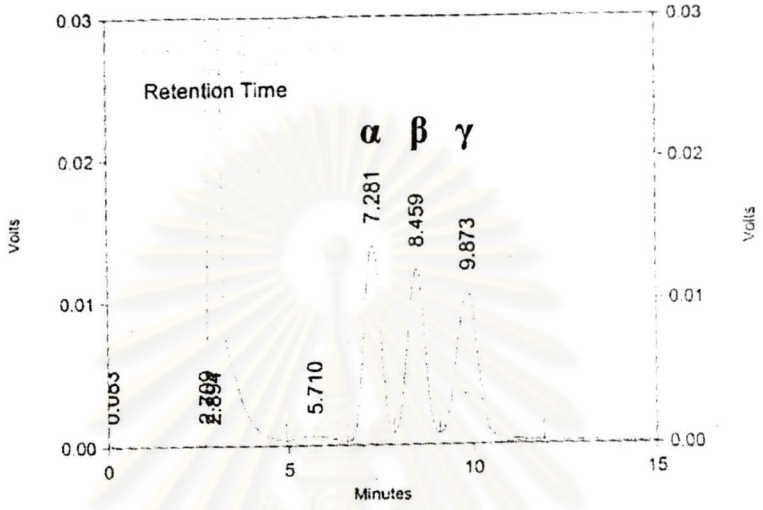


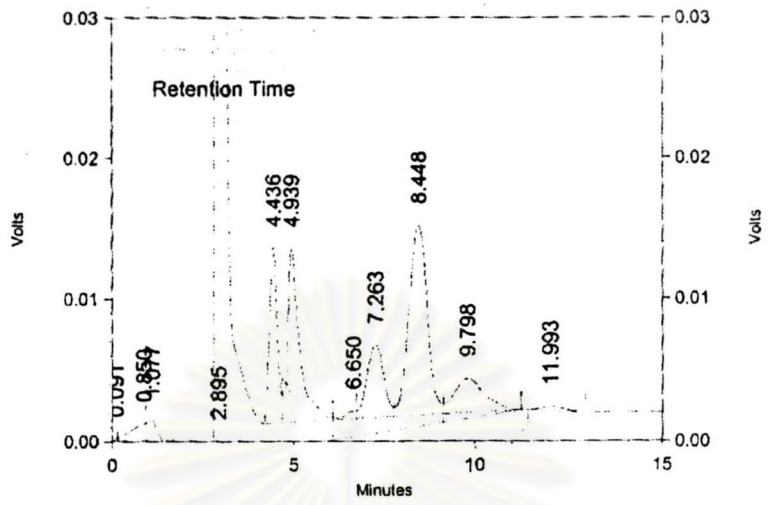
Figure 29. Summary of the iodine test for dextrinizing activity of the wild type and the mutant CGTases. Clear zone surrounding the colonies indicates starch hydrolysis activity.

The cyclodextrin production by the recombinant enzymes at 8 and 12 hours was determined by using HPLC. All recombinants showed the cyclodextrin forming activity. The results were shown in Fig. 30. The recombinants produced β-cyclodextrin as a major product and, hence, were β-CGTases. The pNan4 and 7 had increased proportion of β-cyclodextrin production, while that of α-cyclodextrin production were decreased significantly and there were very little or no γ-cyclodextrin production. The pNan5 and 9 had increased proportion of γ-cyclodextrin production significantly and produced slightly lower proportion of α- and β-cyclodextrins. The pNan6 had increased proportion of β-cyclodextrin production significantly and produced lower proportion of α-cyclodextrin but the γ-cyclodextrin production was not effected. The pNan8 had the higher proportion of β- and γ-cyclodextrin production and produced lower proportion of α-cyclodextrin significantly. The pNan10 had increased proportion of γ-cyclodextrin production significantly, while the production of α-cyclodextrin was decreased significantly but that of β-cyclodextrin was not affected.

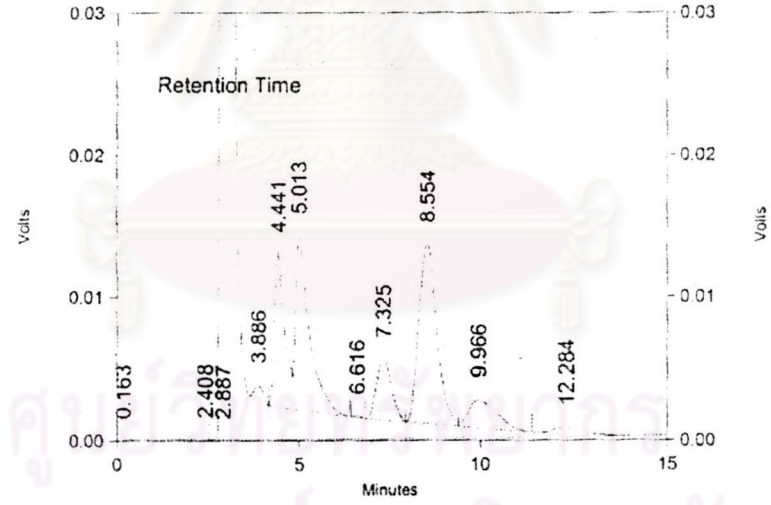


Standard α-,β- and γ-cyclodextrins

Figure 30. HPLC profiles of cyclodextrins formed by the wild type and mutant CGTases.

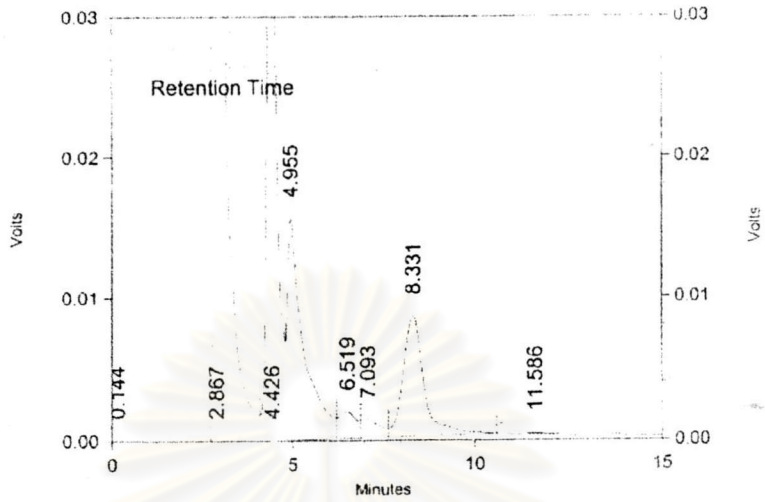


pVR328 (incubation time 8 hours)

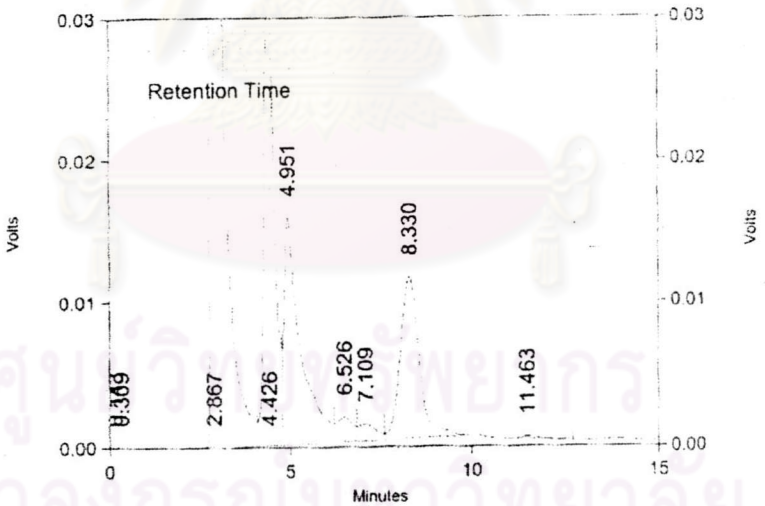


pVR328 (incubation time 12 hours)

Figure 30. (continue) HPLC profiles of cyclodextrins formed by the wild type and mutant CGTases.

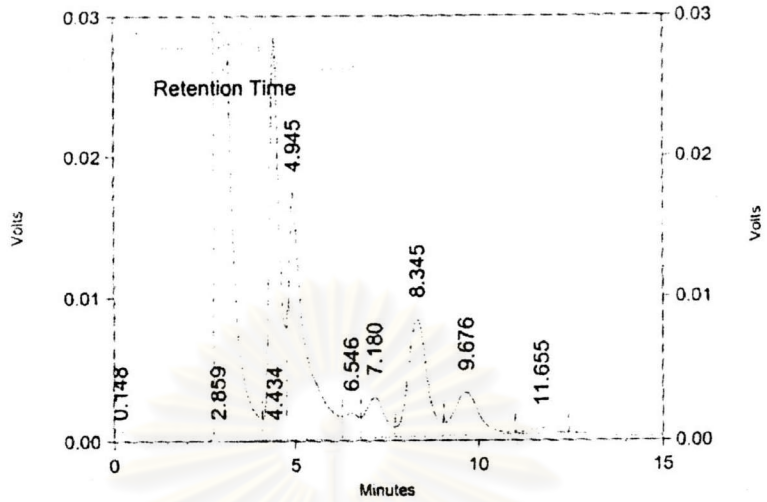


pNan4 (incubation time 8 hours)

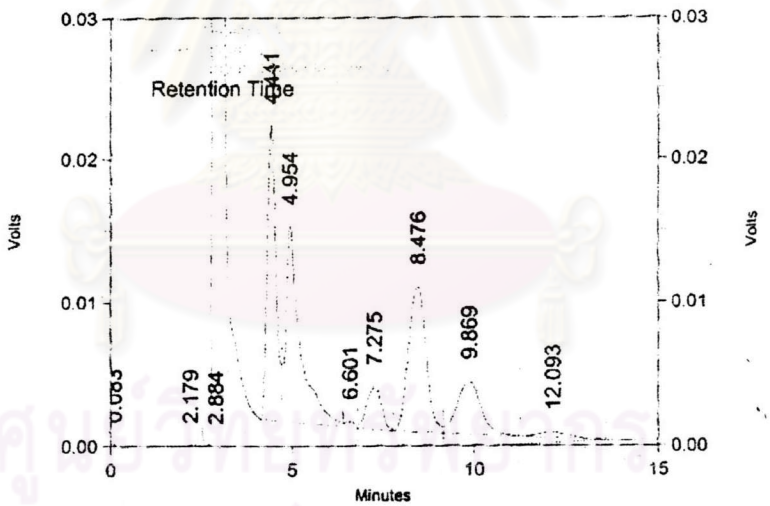


pNan4 (incubation time 12 hours)

Figure 30. (continue) HPLC profiles of cyclodextrins formed by the wild type and mutant CGTases.

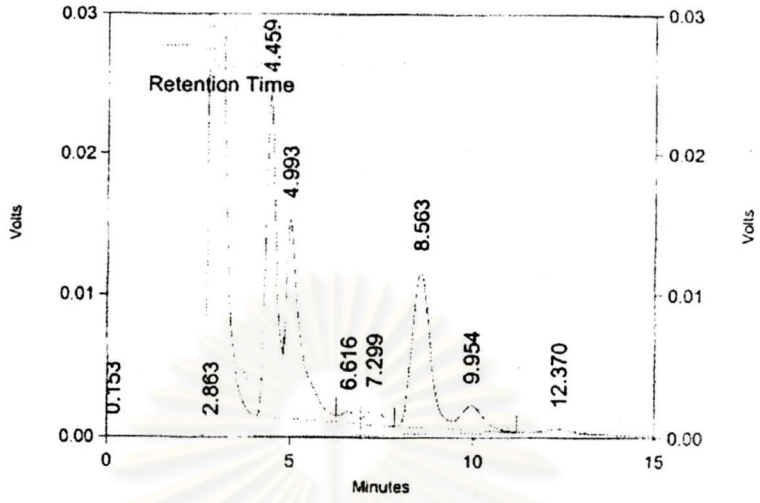


pNan5 (incubation time 8 hours)

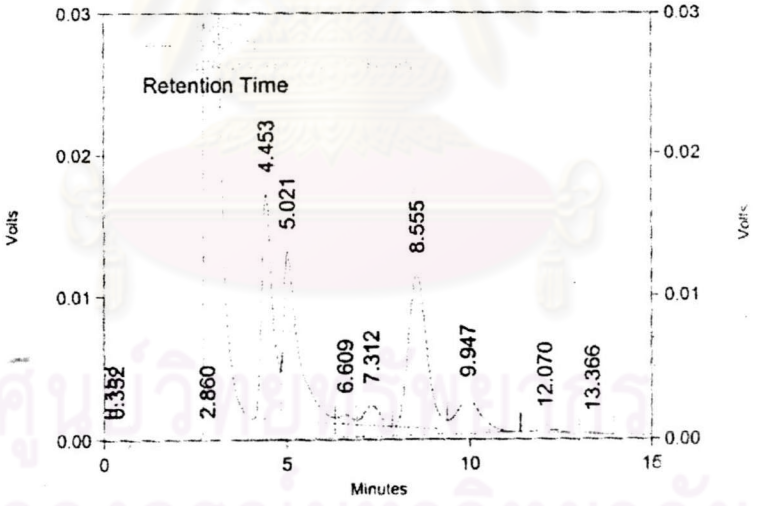


pNan5 (incubation time 12 hours)

Figure 30. (continue) HPLC profiles of cyclodextrins formed by the wild type and mutant CGTases.

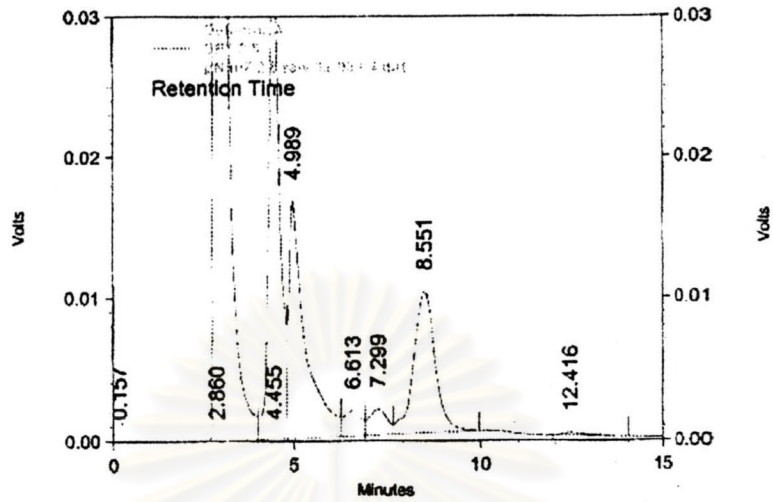


pNan6 (incubation time 8 hours)

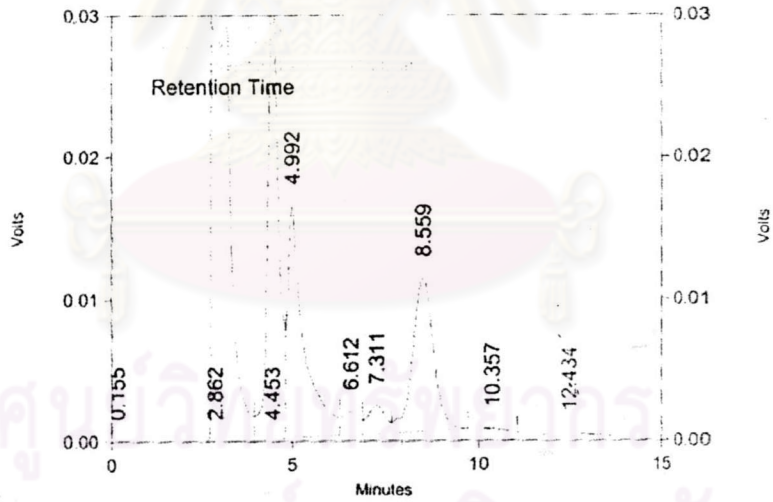


pNan6 (incubation time 12 hours)

Figure 30. (continue) HPLC profiles of cyclodextrins formed by the wild type and mutant CGTases.

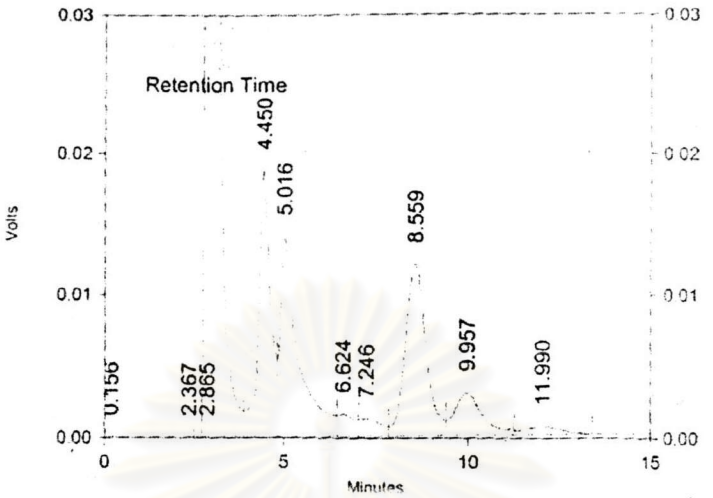


pNan7 (incubation time 8 hours)

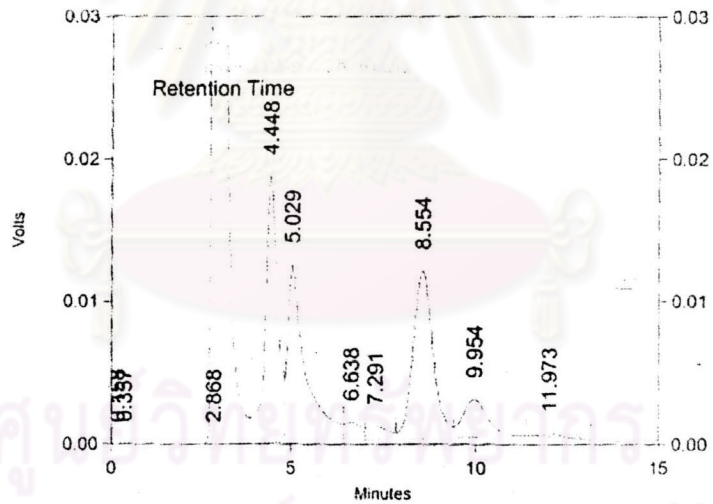


pNan7 (incubation time 12 hours)

Figure 30. (continue) HPLC profiles of cyclodextrins formed by the wild type and mutant CGTases.

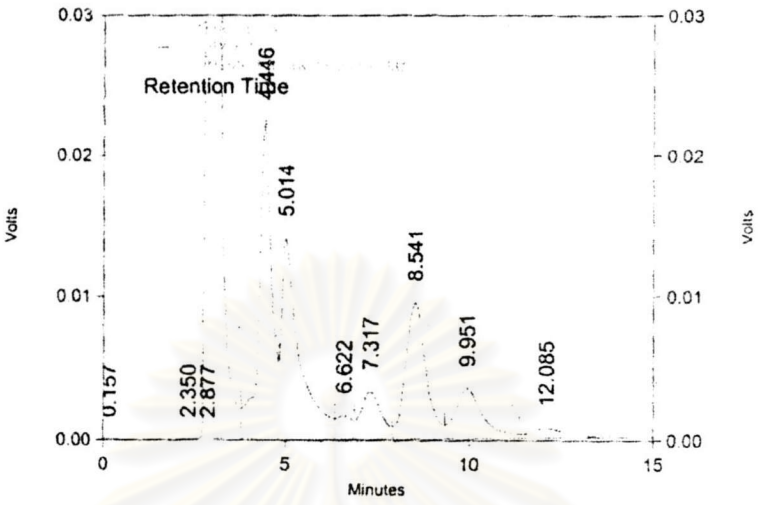


pNan8 (incubation time 8 hours)

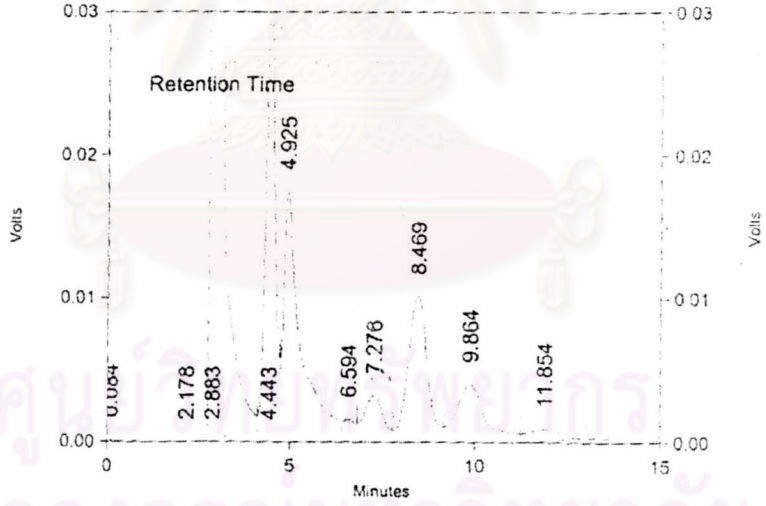


pNan8 (incubation time 12 hours)

Figure 30. (continue) HPLC profiles of cyclodextrins formed by the wild type and mutant CGTases.

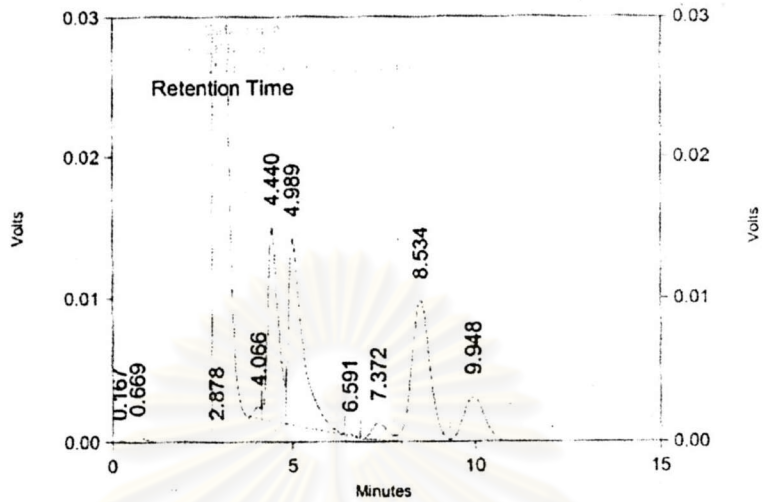


pNan9 (incubation time 8 hours)

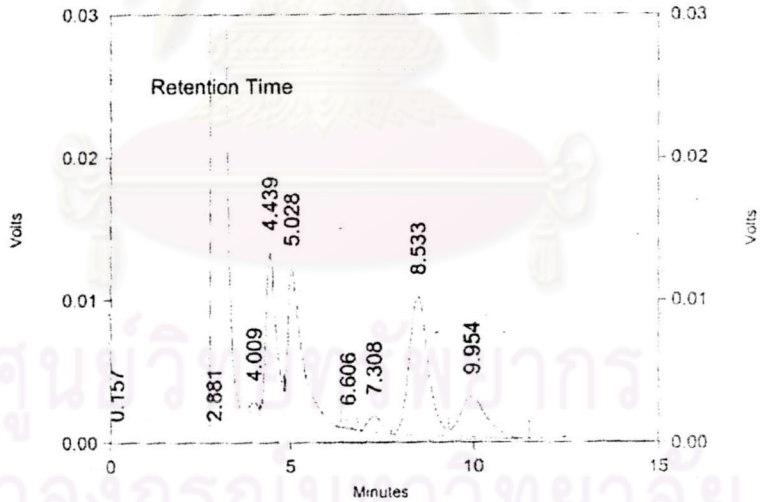


pNan9 (incubation time 12 hours)

Figure 30. (continue) HPLC profiles of cyclodextrins formed by the wild type and mutant CGTases.



pNan10 (incubation time 8 hours)



pNan10 (incubation time 12 hours)

Figure 30. (continue) HPLC profiles of cyclodextrins formed by the wild type and mutant CGTases.

The cyclodextrin ratios were calculated from the peak areas of α-, β- and γ-cyclodextrins in HPLC profiles. The results from Fig. 37 and Table 3 indicated that the reaction times for cyclodextrin forming activity at 8 and 12 hours had no affect in the proportions of cyclodextrins produced. Thus, the reactions at these time points were more or less in the stationary phase for all the recombinant enzymes.

Table 3. Summary of the mutant CGTase activities.

Plasmid	Dextrinizing activity (U/mg protein)*[1]	Cyclodextrin ratios, α:β:γ /total area of cyclodextrin peaks × 10^5 (unit area)	
		Incubation 8 hours*[2]	Incubation 12 hours*[3]
pVR328	1.10	21:64:15 /6.70	22:65:13 /7.76
pNan4	0.36	9:91:0 /3.76	11:88:0 /4.40
pNan5	1.08	13:58:28 /5.44	14:59:27 /5.87
pNan6	0.62	8:73:18 /5.00	9:75:15 /5.26
pNan7	1.27	13:87:0 /4.46	16:84:0 /4.80
pNan8	0.40	8:70:22 /6.01	7:73:20 /6.65
pNan9	0.56	13:58:29 /6.77	14:60:25 /8.00
pNan10	0.67	6:67:27 /5.17	6:68:26 /5.39

*[1] Fuwa method assay and average of 2 separate determonations, *[2] average of 4 separate determinations, *[3]average of 2 separate determinations