CHAPTER III

RESULTS

For cloning and sequencing of the structural gene encoding gene CGTases from 2 strains of *Paenibacillus* sp., the RB01 and T16. *E. coli* JM109 and pGEM®-Teasy vector were used as host and plasmid. CGTase genes were expressed in the transformed *E. coli*. Both recombinant enzymes were purified and characterized in comparison to their native enzymes.

3.1 Cloning of CGTase genes from *Paenibacillus* sp.RB01 and *Paenibacillus* sp.T16

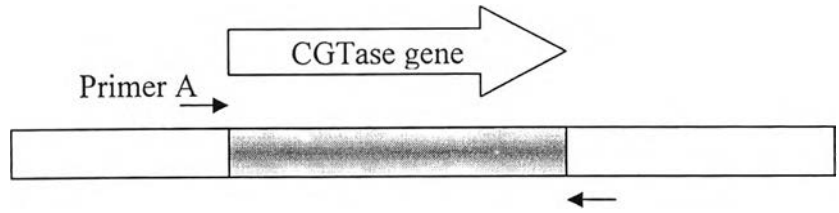
3.1.1 PCR amplification of CGTase gene

From the amino acid and nucleotide sequences of the previous study on *Paenibacillus* sp. A11 *(Bacillus circulans* A11) (**63**), several pairs of oligonucleotide were designed from the conserved amino acid sequences of the CGTase gene and used as probes for the screening of CGTase gene of *Bacilli*. From the analysis of the hybridization blot, nucleotide sequences of RB01 and T16 yielded up to 98% similarity to *B. circulans* A11. Therefore, in this study, new pair of oligonucleotides was designed from the sequences upstream and downstream of the CGTase gene of *B. circulans* A11 to cover the whole CGTase gene. The annealing sites and the sequences of oligonucleotides were shown in Figure 4 (a) and (b). They were used to amplify the CGTase gene fragment from the chromosomal DNA of *Paenibacillus* sp.RB01 and *Paenibacillus* sp.T16. One major band of 2.4 kb which was band of expected size was observed (Figure 4 (c)). *Bacillus liceniformis*, a non-CGTase producing bacillus used as a negative control. The PCR products which contained the CGTase gene could be obtained with this pair of primers for both *Paeinibacillus* sp. RB01 and T16.

3.1.2 DNA cloning and selection of positive colony

The PCR products contained the CGTase genes were run on the 0.7% agarose gel, and the expected band of 2.4 kb was eluted, ligated with the pGEM-Teasy vector and transformed into *E. coli* JM 109. The vector which was inserted with CGTase gene generated white colonies on the culture plate. Clones were analyzed further by techniques, such as expression of their enzymes activities on the selective plate. By incubating for 24 hours, the replica plate of white colony which contains the CGTase gene showed clear zone on the LB plate containing 1% soluble starch after treating with iodine solution or phenolphthalein-methyl orange solution (Figure 6 (a) and (b)). The resulting transformants of *Paenibacillus* sp.RB01 and *Paenibacillus* sp. T16 were named pRB and pT, respectively. The transformants were kept in LB medium containing 30% glycerol at -80°C as stock for the re-cultivation for the expression of CGTase gene and extraction of the plasmid for DNA sequencing. The plasmids were kept at -20°C.

(a)



(b)

Primer B

Primer A	5'- GGCTATGCTTTCCTTACCTTACCC -3'
Primer B	5'-ATAGCACCTTTCCCCCACATAACG -3'

(c)

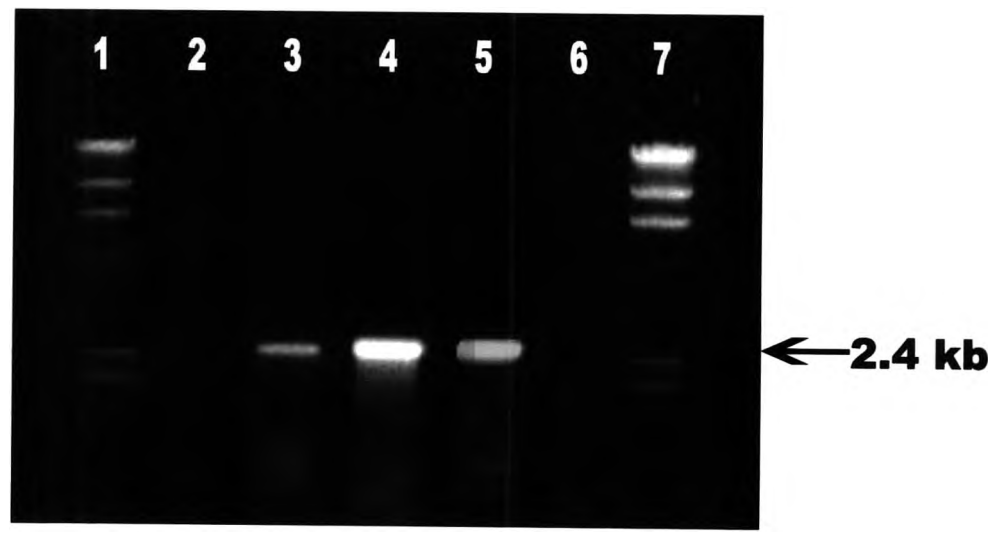


Figure 4 Amplification of the CGTase genes.

(a) The drawing shows the approximate locations of PCR primer annealing sites.

(b) Oligonucleotide primers designed for the amplification of the CGTase genes.

(c) The PCR products were amplified from the reactions using DNA template as followed: Lane 2; no template represented the negative control, Lane 3; pVR316 (a plasmid containing the CGTase gene from *Bacillus circulans* A11) represented the positive control, Lane 4; *Paenibacillus* sp. RB01; Lane 5 *Paenibacillus* sp. T16; Lane 6 *Bacillus licheniformis* SK-1 (a chitinase producing bacteria) represented the negative control. Lanes 1 and 7 were DNA marker, λ /*Hin*d III

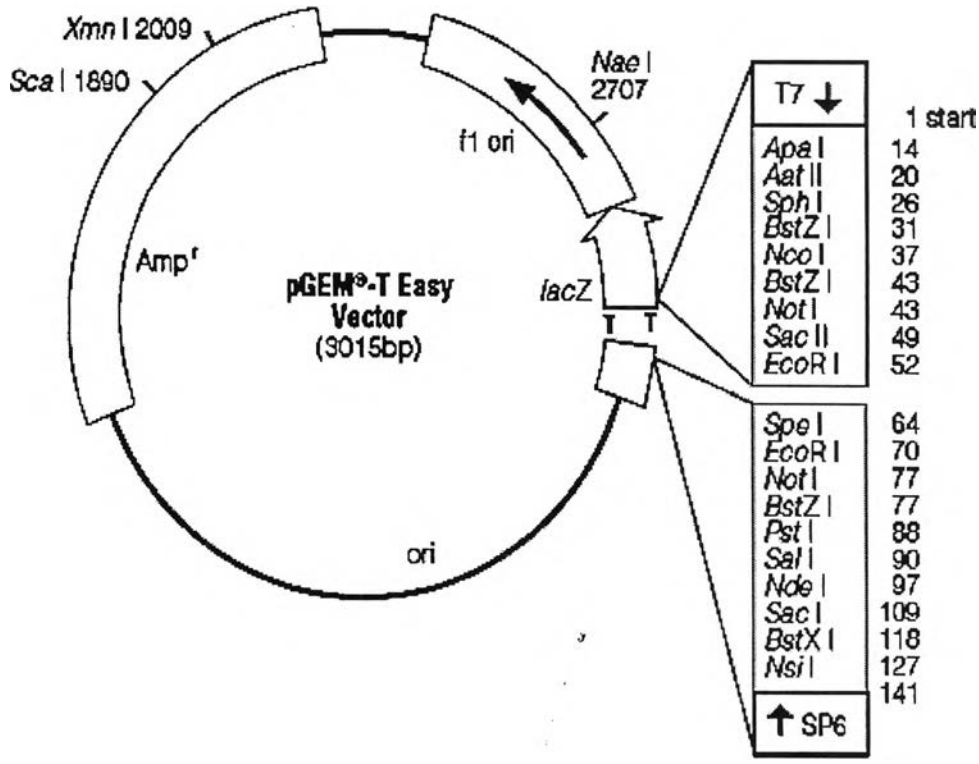


Figure 5 Restriction map of Plasmid pGEM®-T Easy

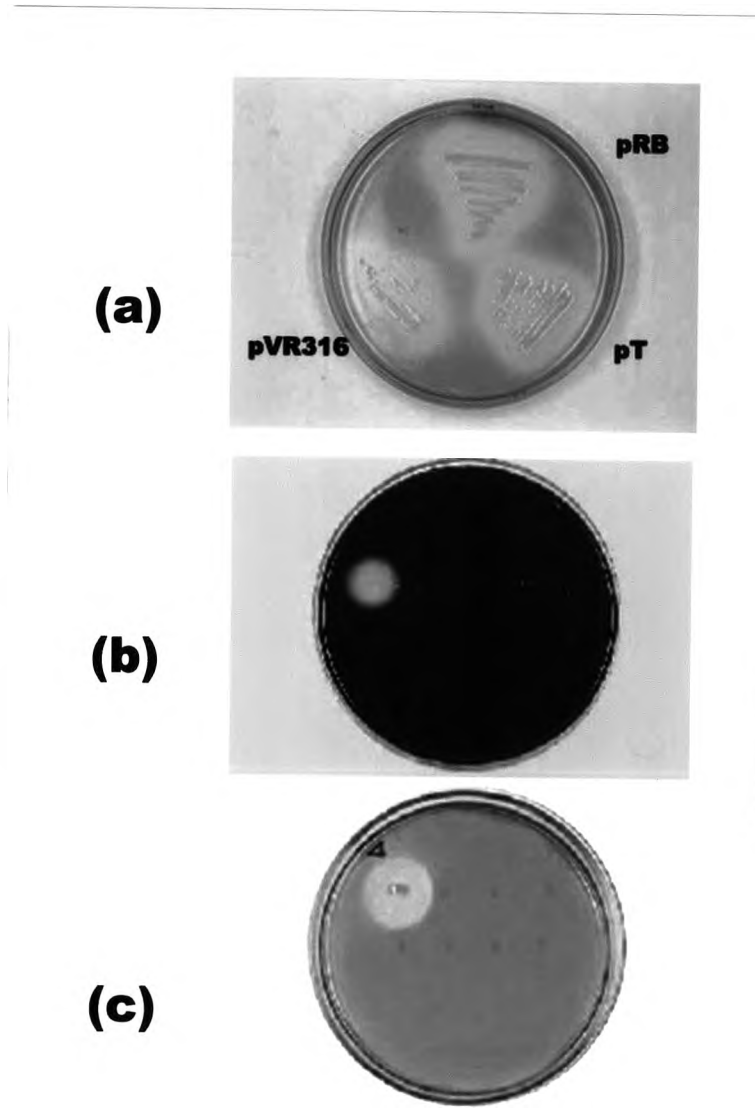


Figure 6 Identification of CGTase producing bacteria

(a) pRB, pTand pVR316 (a transformant containing CGTase gene from *Paenibacillus* sp. A11) on phenolphthalein plates. (b) the recombinant colony of pRB on starch - agar plate containing x - gal and ampicillin after treating with iodine solution. (c) pRB on phenolphthalein – methyl orange plates.

3.2 DNA sequencing

The DNA sequencing was done using the primer M13 forward, primer M13 reverse, primers 1-5 and primer B were used to design additional primers in order to sequence the whole gene. Their annealing sites were shown in Figure7. The DNA sequence showed an open reading frame of 2194 bp encoding 732 amino acid residues for pRB and an open reading frame of 2139 bp encoding 713 amino acid residues for pT (Figures 8 and 9).

Alignment of the deduced amino acid of CGTase from pRB and pT were compared with various published CGTase sequences. The phylogenic tree was built by the neighbor joining method, implemented in the CLUSTALW program (**79**). The results were shown in Figures 10 and 11. The accession number and abbreviation of each published CGTase were listed in Table 4. The amino acid sequences of pRB and pT showed 97% homology among those *Bacilli* used for the alignment. pRB gave 97% identities to the CGTases from A11, 95% to *Bacillus* sp. #1011 and *Bacillus* sp. Strain no.38-2, 85% to *B. circulans* strain 251. pT gave 99% identities to the CGTases from A11, 98% to *Bacillus* sp. #1011, 97% to *Bacillus* sp. Strain no.38-2 and 85% to *B. circulans* strain 251.

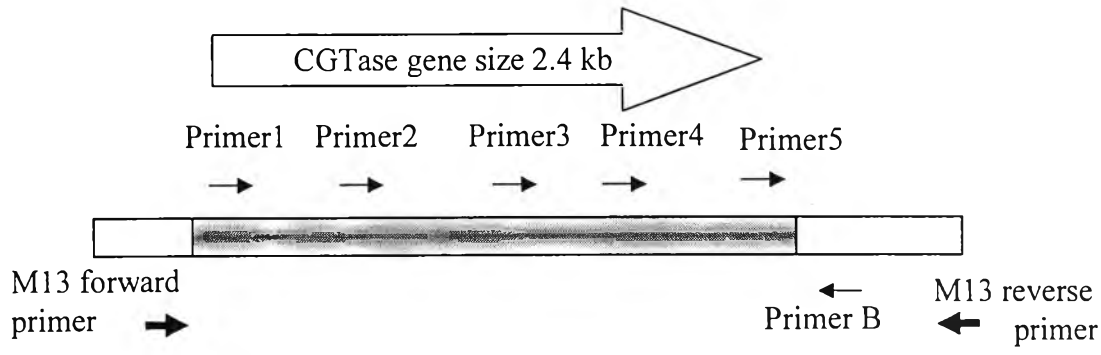


Figure 7 Approximate annealing sites of the sequencing primers along the cloned CGTase gene.

The top arrow indicates the orientation and size of CGTase.

The small arrows locate the primers used for recombinant plasmid sequencing.

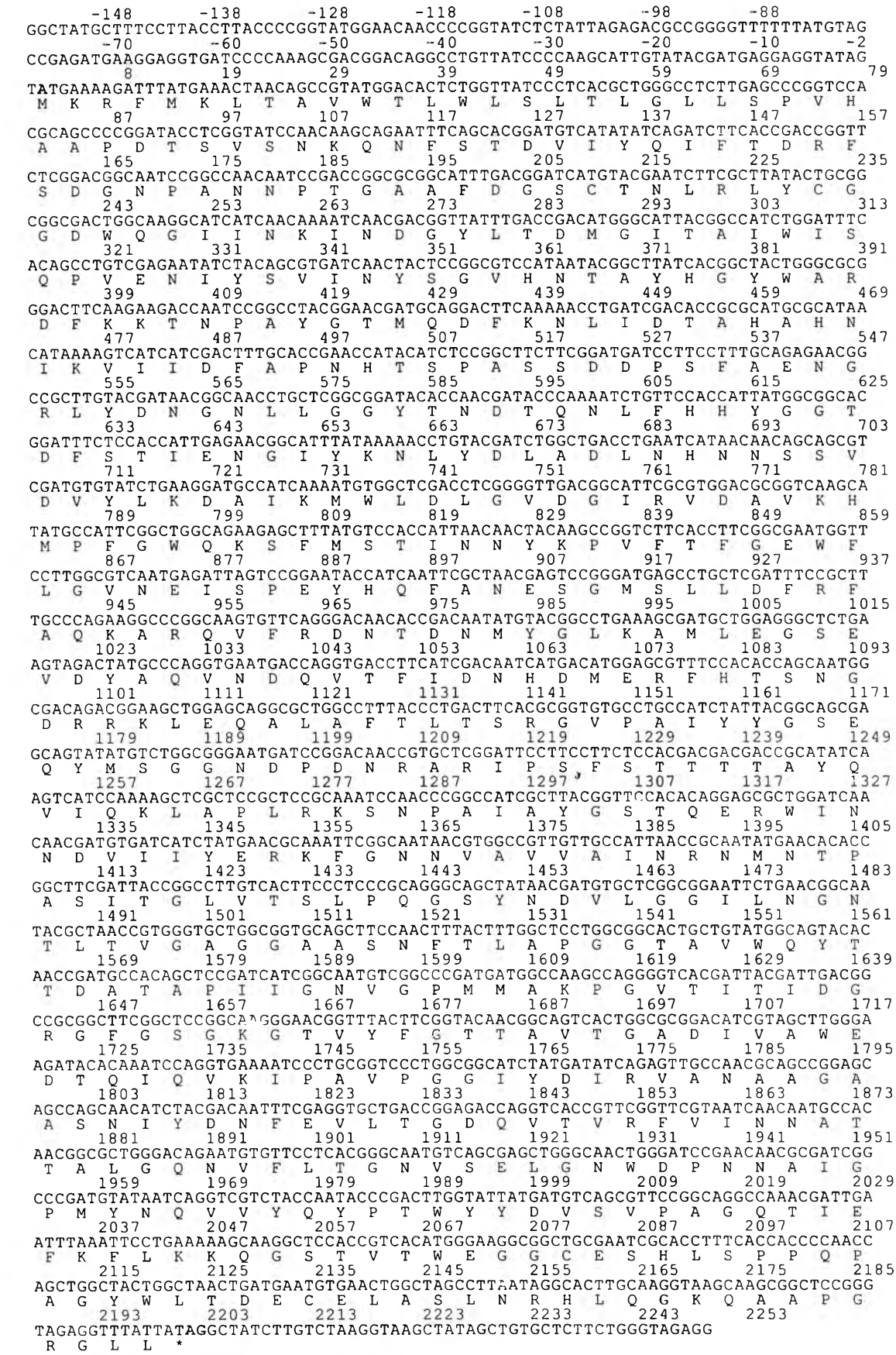


Figure 8 Nucleotide and deduced amino acid sequence of recombinant CGTase of pRB. The nucleotides were numbered. The initiator codon (ATG) of the deduced amino acid residues and the terminator codon (TAG) were also bold letter.

```
      -149       -139       -129       -119       -109        -99        -89        -79
GGCTATGCTTTCCTTACCTTACCCCGGTATGGAACAACCCCGGTATCTCTATTAGAGACGCCGGGGTTTTTTATGTAG
       -71        -61        -51        -41        -31        -21        -11         -3
CCGAGATGAAGGAGGTGATCCCCAAAGCGACGGACAGGCCTGTTATCCCCAAGCATTGTATACGATGAGGAGGTATAT
         8         18         28         38         48         58         68         78
GTATGAAAAGATTTATGAAACTAACAGCCGTATGGACACTCTGGTTATCCCTCACGCTGGGCCTCTTGAGCCCGGTCC
  M  K  R  F  M  K  L  T  A  V  W  T  L  W  L  S  L  T  L  G  L  L  S  P  V  H
        86         96        106        116        126        136        146        156
ACGCAGCCCCGGATACCTCGGTATCCAACAAGCAGAATTTCAGCACGGATGTCATATATCAGATCTTCACCGACCGGT
  A  A  P  D  T  S  V  S  N  K  Q  N  F  S  T  D  V  I  Y  Q  I  F  T  D  R  F
       164        174        184        194        204        214        224        234
TCTCGGACGGCAATCCGGCCAACAATCCGACCGGCGCGGCATTTGACGGATCATGTACGAATCTTCGCTTATACTGCG
  S  D  G  N  P  A  N  N  P  T  G  A  A  F  D  G  S  C  T  N  L  R  L  Y  C  G
       242        252        262        272        282        292        302        312
GCGGCGACTGGCAAGGCATCATCAACAAAATCAACGACGGTTATTTGACCGGCATGGGCATTACGGCCATCTGGATTT
  G  D  W  Q  G  I  I  N  K  I  N  D  G  Y  L  T  G  M  G  I  T  A  I  W  I  S
       320        330        340        350        360        370        380        390
CACAGCCTGTCGAGAATATCTACAGCGTGATCAACTACTCCGGCGTCCATAATACGGCTTATCACGGCTACTGGGCGC
  Q  P  V  E  N  I  Y  S  V  I  N  Y  S  G  V  H  N  T  A  Y  H  G  Y  W  A  R
       398        408        418        428        438        448        458        468
GGGACTTCAAGAAGACCAATCCGGCCTACGGAACGATGCAGGACTTCAAAAACCTGATCGACACCGCGCATGCGCATA
  D  F  K  K  T  N  P  A  Y  G  T  M  Q  D  F  K  N  L  I  D  T  A  H  A  H  N
       476        486        496        506        516        526        536        546
ACATAAAAGTCATCATCGACTTTGCACCGAACCATACATCTCCGGCTTCTTCGGATGATCCTTCCTTTGCAGAGAACG
  I  K  V  I  I  D  F  A  P  N  H  T  S  P  A  S  S  D  D  P  S  F  A  E  N  G
       554        564        574        584        594        604        614        624
GTCGCTTGTACGATAACGGCAACCTGCTCGGCGGATACACCAACGATACCCAAAATCTGTTCCACCATTATGGCGGCA
  R  L  Y  D  N  G  N  L  L  G  G  Y  T  N  D  T  Q  N  L  F  H  H  Y  G  G  T
       632        642        652        662        672        682        692        702
CGGATTTCTCCACCATTGAGAACGGCATTTATAAAAACCTGTACGATCTGGCTGACCTGAATCATAACAACAGCAGCG
  D  F  S  T  I  E  N  G  I  Y  K  N  L  Y  D  L  A  D  L  N  H  N  N  S  S  V
       710        720        730        740        750        760        770        780
TCGATGTGTATCTGAAGGATGCCATCAAAATGTGGCTCGACCTCGGGGTTGACGGCATTCGCGTGGACGCGGTCAAGC
  D  V  Y  L  K  D  A  I  K  M  W  L  D  L  G  V  D  G  I  R  V  D  A  V  K  H
       788        798        808        818        828        838        848        858
ATATGCCATTCGGCTGGCAGAAGAGCTTTATGTCCACCATTAACAACTACAAGCCGGTCTTCACCTTCGGCGAATGGT
  M  P  F  G  W  Q  K  S  F  M  S  T  I  N  N  Y  K  P  V  F  T  F  G  E  W  F
       866        876        886        896        906        916        926        936
TCCTTGGCGTCAATGAGATTAGTCCGGAATACCATCAATTCGCTAACGAGTCCGGGATGAGCCTGCTCGATTTCCGCT
  L  G  V  N  E  I  S  P  E  Y  H  Q  F  A  N  E  S  G  M  S  L  L  D  F  R  F
       944        954        964        974        984        994       1004       1014
TTGCCCAGAAGGCCCGGCAAGTGTTCAGGGACAACACCGACAATATGTACGGCCTGAAAGCGATGCTGGAGGGCTCTG
  A  Q  K  A  R  Q  V  F  R  D  N  T  D  N  M  Y  G  L  K  A  M  L  E  G  S  E
      1022       1032       1042       1052       1062       1072       1082       1092
AAGTAAACTATGCCCAGGTGAATGACCAGGTGACCTTCATCGACAATCATGACATGGAGCGTTTCCACACCAGCAATG
  V  N  Y  A  Q  V  N  D  Q  V  T  F  I  D  N  H  D  M  E  R  F  H  T  S  N  G
      1100       1110       1120       1130       1140       1150       1160       1170
GCGACAGACGGAAGCTGGAGCAGGCGCTGGCCTTTACCCTGACTTCACGCGGTGTGCCTGCCATCTATTACGGCAGCG
  D  R  R  K  L  E  Q  A  L  A  F  T  L  T  S  R  G  V  P  A  I  Y  Y  G  S  E
      1178       1188       1198       1208       1218       1228       1238       1248
AGCAGTATATGTCTGGCGGGAATGATCCGGACAACCGTGCTCGGATTCCTTCCTTCTCCACGACGACGACCGCATATC
  Q  Y  M  S  G  G  N  D  P  D  N  R  A  R  I  P  S  F  S  T  T  T  T  A  Y  Q
      1256       1266       1276       1286       1296       1306       1316       1326
AAGTCATCCAAAAGCTCGCTCCGCTCCGCAAATCCAACCCGGCCATCGCTTACGGTTCCACACAGGAGCGCTGGATCA
  V  I  Q  K  L  A  P  L  R  K  S  N  P  A  I  A  Y  G  S  T  Q  E  R  W  I  N
      1334       1344       1354       1364       1374       1384       1394       1404
ACAACGATGTGATCATCTATGAACGCAAATTCGGCAATAACGTGGCCGTTGTTGCCATTAACCGCAATATGAACACAC
  N  D  V  I  I  Y  E  R  K  F  G  N  N  V  A  V  V  A  I  N  R  N  M  N  T  P
      1412       1422       1432       1442       1452       1462       1472       1482
CGGCTTCGATTACCGGCCTTGTCACTTCCCTCCCGCAGGGCAGCTATAACGATGTGCTCGGCGGAATTCTGAACGGCA
  A  S  I  T  G  L  V  T  S  L  P  Q  G  S  Y  N  D  V  L  G  G  I  L  N  G  N
      1490       1500       1510       1520       1530       1540       1550       1560
ATACGCTAACCGTGGGTGCTGGCGGTGCAGCTTCCAACTTTACTTTGGCTCCTGGCGGCACTGCTGTATGGCAGTACA
  T  L  T  V  G  A  G  G  A  A  S  N  F  T  L  A  P  G  G  T  A  V  W  Q  Y  T
      1568       1578       1588       1598       1608       1618       1628       1638
CAACCGATGCCACAGCTCCGATCATCGGCAATGTCGGCCCGATGATGGCCAAGCCAGGGGTCACGATTACGATTGACG
  T  D  A  T  A  P  I  I  G  N  V  G  P  M  M  A  K  P  G  V  T  I  T  I  D  G
      1646       1656       1666       1676       1686       1696       1706       1716
GCCGCGGCTTCGGCTCCGGCAAGGGAACGGTTTACTTCGGTACAACGGCAGTCACTGGCGCGGACATCGTAGCTTGGG
  R  G  F  G  S  G  K  G  T  V  Y  F  G  T  T  A  V  T  G  A  D  I  V  A  W  E
      1724       1734       1744       1754       1764       1774       1784       1794
AAGATACACAAATCCAGGTGAAAATCCCTGCGGTCCCTGGCGGCATCTATGATATCAGAGTTGCCAACGCAGCCGGAG
  D  T  Q  I  Q  V  K  I  P  A  V  P  G  G  I  Y  D  I  R  V  A  N  A  A  G  A
      1802       1812       1822       1832       1842       1852       1862       1872
CAGCCAGCAACATCTACGACAATTTCGAGGTGCTGACCGGAGACCAGGTCACCGTTCGGTTCGTAATCAACAATGCCA
  A  S  N  I  Y  D  N  F  E  V  L  T  G  D  Q  V  T  V  R  F  V  I  N  N  A  T
      1880       1890       1900       1910       1920       1930       1940       1950
CAACGGCGCTGGGACAGAATGTGTTCCTCACGGGCAATGTCAGCGAGCTGGGCAACTGGGATCCGAACAACGCGATCG
  T  A  L  G  Q  N  V  F  L  T  G  N  V  S  E  L  G  N  W  D  P  N  N  A  I  G
      1958       1968       1978       1988       1998       2008       2018       2028
GCCCGATGTATAATCAGGTCGTCTACCAATACCCGACTTGGTATTATGATGTCAGCGTTCCGGCAGGCCAAACGATTG
  P  M  Y  N  Q  V  V  Y  Q  Y  P  T  W  Y  Y  D  V  S  V  P  A  G  Q  T  I  E
      2036       2046       2056       2066       2076       2086       2096       2106
AATTTAAATTCCTGAAAAAGCAAGGCTCCACCGTCACATGGGAAGGCGGCGCGAATCGCACCTTCACCACCCCAACCA
  F  K  F  L  K  K  Q  G  S  T  V  T  W  E  G  G  A  N  R  T  F  T  T  P  T  S
      2114       2124       2134       2144       2154       2164       2174       2184
GCGGCACGGCAACTATGAATGTGAACTGGCTAGCCTAATAGGCACTTGCAAGGTAAGCAAGCGGCTCCGGGTAGAGGC
  G  T  A  T  M  N  V  N  W  L  A  *
      2192       2202       2212       2222       2232
TCGGGGCCGCTTGTTACGTTATGTGGGGGAAAGGTGCTAT
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Figure 9 Nucleotide and deduced amino acid sequence of recombinant CGTase of pT. The nucleotides were numbered. The initiator codon (ATG) of the deduced amino acid residues and the terminator codon (TAA) were also bold letter.

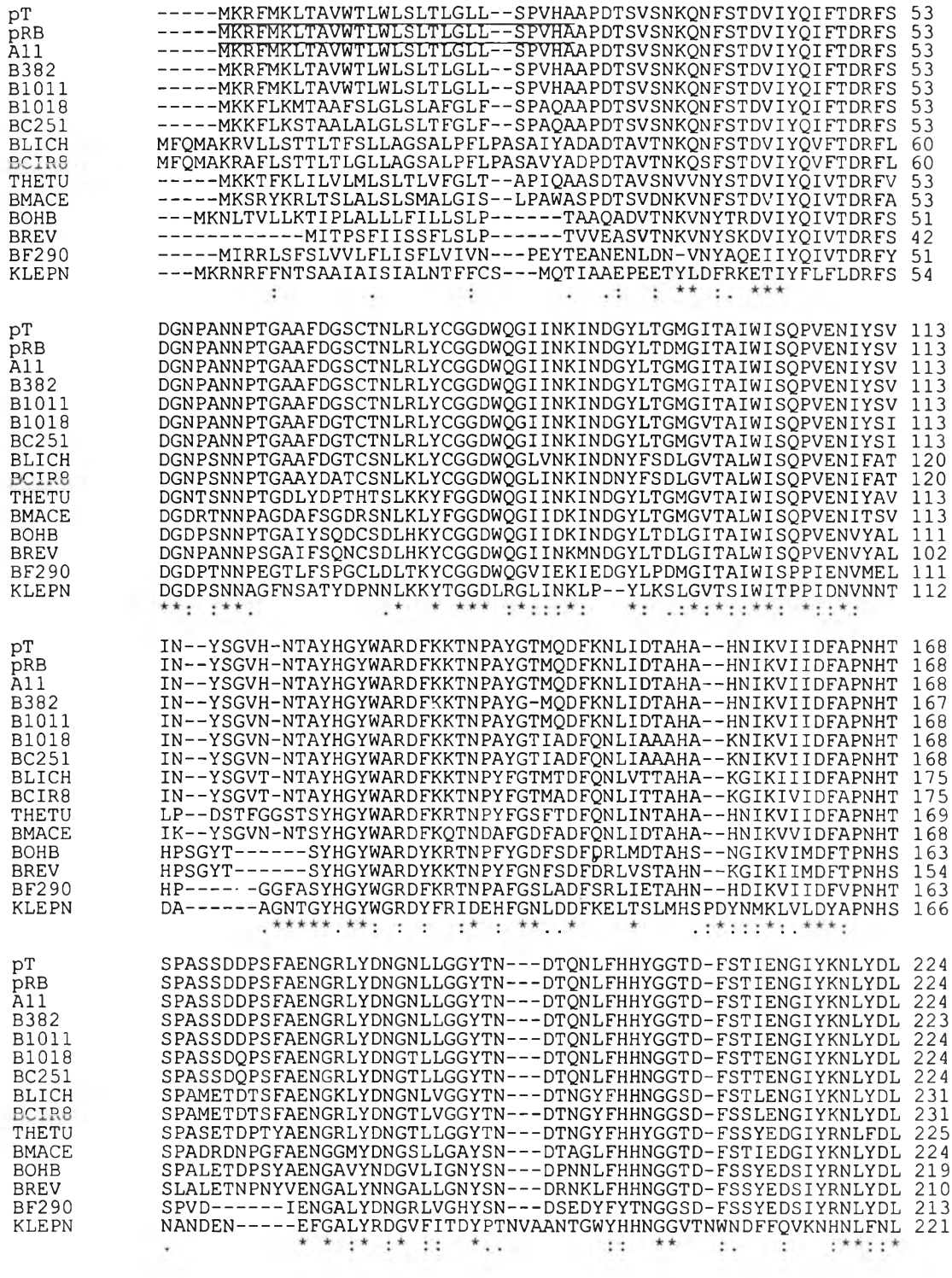

Figure 10 Comparison of the deduced amino acid sequences of CGTase from pRB and pT. The amino acid residues indicated by asterisks (*) were completely identical or conserved in all CGTases. The amino acid residues indicated by colon (:) were conserved and dot (.) among the CGTase. Dashes (---) denote gaps. Underline letters denote the signal (leader region) of CGTase.

```
pT          ADLNHNNSSVDVYLKDAIKMWLDLGVDGIRVDAVKHMPFGWQKSFMSTINN------YKP 278
pRB         ADLNHNNSSVDVYLKDAIKMWLDLGVDGIRVDAVKHMPFGWQKSFMSTINN------YKP 278
A11         ADLNHNNSSVDVYLKDAIKMWLDLGVDGIRVDAVKHMPFGWQKSFMSTINN------YKP 278
B382        ADLNHNNSSVDVYLKDAIKMWLDLGVDGIRVDAVKHMPFGWQKSFMSTINN------YKP 277
B1011       ADLNHNNSSVDVYLKDAIKMWLDLGVDGIRVDAVKHMPFGWQKSFMATINN------YKP 278
B1018       ADLNHNNSTSDVYLKDAIKMWLDLGIDGIRMDAVKHMPFGWQKSFMAAVNN------YKP 278
BC251       ADLNHNNSTVDVYLKDAIKMWLDLGIDGIRMDAVKHMPFGWQKSFMAAVNN------YKP 278
BLICH       ADLNHNNSTIDTYFKDAIKLWLDMGVDGIRVDAVKHMPQGWQKNWMSSIYA------HKP 285
BCIR8       ADFNHNNATIDKYFKDAIKLWLDMGVDGIRVDAVKHMPLGWQKSWMSSIYA------HKP 285
THETU       ADLNQQNSTIDSYLKSAIKVWLDMGIDGIRLDAVKHMPFGWQKNFMDSILS------YRP 279
BMACE       ADINHNNNAMDAYFKSAIDLWLGMGVDGIRFDAVKHMPFGWQKSFVSSIYGG-----DHP 279
BOHB        ADYDLNNTVMDQYLKESIKLWLDKGIDGIRVDAVKHMSEGWQTSLMSDIYA------HEP 273
BREV        ADYDLNNKVVDQYLKESIKLWLIK-IDGIRVDAVKHMSEGWQTSLMSDIYT------YKP 263
BF290       ASLNQQNSFIDRYLKESIQMWLDLGIDGIRVDAVAHMPVGWQKNFVSSIYD------YNP 267
KLEPN       SDLNQSNTDVYQYLLDGSKFWIDAGVDAIRIDAIKHMDKSFIQKWTSDIYDYSKSIGREG 281
            :. : .*     *: .. ..*:   :*.**.**: **  .:  .    :          .

pT          VFTFGEWFLGVN----EISPEYHQFANESGMSLLDFRFAQKARQVFRDNT-DNMYGLKAM 333
pRB         VFTFGEWFLGVN----EISPEYHQFANESGMSLLDFRFAQKARQVFRDNT-DNMYGLKAM 333
A11         VFTFGEWFLGVN----EISPEYHQFANESGMSLLDFRFAQKARQVFRDNT-DNMYGLKAM 333
B382        VFNFGEWFLGVN----EISPEYHQFANESGMSLLDFPFAQKARQVFRDNT-DNMYGLKAM 332
B1011       VFTFGEWFLGVN----EISPEYHQFANESGMSLLDFRFAQKARQVFRDNT-DNMYGLKAM 333
B1018       VFTFGEWFLGVN----EVGPENHKFANESGMSLLDFRFAQKVRQVFRDNT-DNMYGLKAM 333
BC251       VFTFGEWFLGVN----EVSPENHKFANESGMSLLDFRFAQKVRQVFRDNT-DNMYGLKAM 333
BLICH       VFTFGEWFLGSA----APDADNTDFANESGMSLLDFRFNSAVRNVFRDNT-SNMYALDSM 340
BCIR8       VFTFGEWFLGSA----ASDADNTDFANKSGMSLLDFRFNSAVRNVFRDNT-SNMYALDSM 340
THETU       VFTFGEWFLGTN----EIDVNNTYFANESGMSLLDFRFSQKVRQVFRDNT-DTMYGLDSM 334
BMACE       VFTFGEWYLGAD----QTDGDNIKFANESGMNLLDFEYAQEVREVFRDKT-ETMKDLYEV 334
BOHB        VFTFGEWFLGSG----EVDPQNHHFANESGMSLLDFQFGQTIRDVLMDGS-SNWYDFNEM 328
BREV        VFTFGEWFLGTG----EVDPQNHHFANESGMSLLDFQFGQTIRSVLKDRT-SNWYDFNEM 318
BF290       VFTFGEWFTGAG----GSD-EYHYFINNSGMSALDFRYAQVVQDVLRNND-GTMYDLETV 321
KLEPN       FFFFGEWFGASANTTTGVDGNAIDYANTSGSALLDFGFRDTLERVLVGRSGNTMKTLNSY 341
            .* ****: .        . :   : * **   *** : .  . *: .   .   :

pT          LEGSEVNYAQVNDQVTFIDNHDMER---------------------FHTSNGDRRKLEQA 372
pRB         LEGSEVDYAQVNDQVTFIDNHDMER---------------------FHTSNGDRRKLEQA 372
A11         LEGSEVDYAQVNDQVTFIDNHDMER---------------------FHTSNGDRRKLEQA 372
B382        LEGSEVDYAQVNDQVTFIDNHDMER---------------------FHTSNGDRRKLEQA 371
B1011       LEGSEVDYAQVNDQVTFIDNHDMER---------------------FHTSNGDRRKLEQA 372
B1018       LEGSAADYAQVDDQVTFIDNHDMER---------------------FHASNANRRKLEQA 372
BC251       LEGSAADYAQVDDQVTFIDNHDMER---------------------FHASNANRRKLEQA 372
BLICH       LTATAADYNQVNDQVTFIDNHDMDR---------------------FKTSAVNNRRLEQA 379
BCIR8       INSTATDYNQVNDQVTFIDNHDMDR---------------------FKTSAVNNRRLEQA 379
THETU       IQSTASDYNFINDMVTFIDNHDMDR---------------------FYN-GGSTRPVEQA 372
BMACE       LASTESQYDYINNMVTFIDNHDMDR---------------------FQVAGSGTRATEQA 373
BOHB        IASTEEDYDEVIDQVTFIDNHDMSR---------------------FSFEQSSNRHTDIA 367
BREV        IKSTEKDYDEVIDQVTFIDNHDMSR---------------------FSMVVFN-FQTDIA 356
BF290       LRETESVYEKPQDQVTFIDNHDINR---------------------FSRNGHSTRTTDLG 360
KLEPN       LIKRQTVFTSDDWQVVFMDNHDMARIGTALRSNATTFGPGNNETGGSQSEAFAQKRIDLG 401
            :     :     *.*:****: *                         : .

pT          LAFTLTSRGVPAIYYGSEQYMS---------GGNDPDNRARIPSFSTTTTAYQVIQKLAP 423
pRB         LAFTLTSRGVPAIYYGSEQYMS---------GGNDPDNRARIPSFSTTTTAYQVIQKLAP 423
A11         LAFTLTSRGVPAIYYGSEQYMS---------GGNDPDNRARIPSFSTTTTAYQVIQKLAP 423
B382        LAFTLTSRGVPAIYYGSEQYMS---------GGNDPDNRARIPSFSTTTTAYQVIQKLAP 422
B1011       LAFTLTSRGVPAIYYGSEQYMS---------GGNDPDNRARLPSFSTTTTAYQVIQKLAP 423
B1018       LAFTLILARVPAIYYGTEQYMS---------GGTDPDNRARIPSFSTSTTAYQVIQKLAP 423
BC251       LAFTLTSRGVPAIYYGTEQYMS---------GGTDPDNRARIPSFSTSTTAYQVIQKLAP 423
BLICH       LAFTLTSRGVPAIYYGTEQYLT---------GNGDPDNRGKMPSFSKSTTAFNVISKLAP 430
BCIR8       LAFTLTSRGVPAIYYGTEQYLT---------GNGDPDNRAKMPSFSKSTTAFNVISKLAP 430
THETU       LAFTLTSRGVPAIYYGTEQYMT---------GNGDPYNRAMMTSFNTSTTAYNVIKKLAP 423
BMACE       LALTLTSRGVPAIYYGTEQYMT---------GDGDPNNRAMMTSFNTGTTAYKVIQALAP 424
BOHB        LAVLLTSRGVPTIYYGTEQYLT---------GGNDPENRKPMSDFDRTTNSYQIISTLAS 418
BREV        LAVLLTSRGVPTIYYGTEQYLT---------GGNDPDNRKPMKTFDRSTNSYKITSKLAS 407
BF290       LAFLLTSRGVPTIYYGTEIYMT---------GDGDPDNRKMMNTFDQSTVAYQIIQQLSS 411
KLEPN       LVATMTVRGIPAIYYGTEHYAANFTSNSFGQVGSDPYNREKMPGFDTESEAFSIIKTLGD 461
            *.  :    :*:****:* * :          . ** **  :  *.  : ::.: . *.

pT          LRKSNPAIAYGSTQERWINNDVIIYERKFGNNVAVVAINRNMNTPASITGLVTSLPQGSY 483
pRB         LRKSNPAIAYGSTQERWINNDVIIYERKFGNNVAVVAINRNMNTPASITGLVTSLPQGSY 483
A11         LRKSNPAIAYGSTQERWINNDVIIYERKFGNNVAVVAINRNMNTPASITGLVTSLPQGSY 483
B382        LRKSNPAIAYGSTQERWINNDVIIYERKFGNNVAVVAINRNMNTPASITGLVTSLPQGSY 482
B1011       LRKSNPAIAYGSTHERWINNDVIIYERKFGNNVAVVAINRNMNTPASITGLVTSLRRASY 483
B1018       LRKSNPAIAYGSTQERWINNDVLIYERKFGSNVAVVAVNRNLNAPASISGLVTSLPQGSY 483
BC251       LRKCNPAIAYGSTQERWINNDVLIYERKFGSNVAVVAVNRNLNAPASISGLVTSLPQGSY 483
BLICH       LRKSNPAIAYGSTQQRWINNDVYIYERKFGKSVAVVAVNRNLTTPTSITNLNTSLPSGTY 490
BCIR8       LRKSNPAIAYGSTQQRWINNDVYVYERKFGKSVAVVAVNRNLSTSASITGLSTSLPTGSY 490
THETU       LRKSNPAIAYGTTQQRWINNDVYIYERKFGNNVALVAINRNLSTSYNITGLYTALPAGTY 483
BMACE       LRKSNPAIAYGTTTERWVNNDVLIIERKFGSSAALVAINRNSSAAYPISGLLSSLPAGTY 484
BOHB        LRQNNPALGYGNTSERWINSDVYIYERSFGDSVVLTAVNSG-DTSYTINNLNTSLPQGQY 477
BREV        LRQRNSALGYGNTTERWINSDVYIYERKFGNSIVLTAVNSS-NRNQTISNLNTSLPQGNY 466
BF290       LRQENRAIAYGDTTERWINEDVFIYERSFNGEYALIAVNRSLNHSYQISSLVTDMPSQLY 471
KLEPN       LRKSSPAIQNGTYTELWVNDDILVFERRSGNDIVIVALNRGEANTINVK--NIAVPNGVY 519
            **: . *:  *   : *:*.*: : **  . . .: *:* .      :.    :    *
```

Figure 10 (cont'd)

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pT          NDVLGGILNGNTLTVGAGGAASNFTLAPGGTAVWQYTT-DATAPIIGNVGPMMAKPGVTI 542
pRB         NDVLGGILNGNTLTVGAGGAASNFTLAPGGTAVWQYTT-DATAPIIGNVGPMMAKPGVTI 542
A11         NDVLGGILNGNTLTVGAGGAASNFTLAPGGTAVWQYTT-DATAPIIGNVGPMMAKPGVTI 542
B382        NDVLGGILNGNTLTVGAGGAASNFTLAPGGTAVWQYTT-DATAPINGNVGPMMAKAGVTI 541
B1011       NDVLGGILNGNTLTVGAGGAASNFTLAPGGTAVWQYTT-DATTPIIGNVGPMMAKPGVTI 542
B1018       NDVLGGLLNGNTLTVGSGGAASNFTLAAGGTAVWQYTA-ATATPTIGHVGPMMAKPGVTI 542
BC251       NDVLGGLLNGNTLSVGSGGAASNFTLAAGGTAVWQYTA-ATATPTIGHVGPMMAKPGVTI 542
BLICH       TDVLGGVLNGNNITS-SGGNISSFTLAAGATAVWQYTA-SETTPTIGHVGPVMGKPGNVV 548
BCIR8       TDVLGGVLNGNNITS-TNGSINNFTLAAGATAVWQYTT-AETTPTIGHVGPVMGKPGNVV 548
THETU       TDVLGGLLNGNSISVASDGSVTPFTLSAGEVAVWQYVS-SSNSPLIGHVGPTMTKAGQTI 542
BMACE       SDVLNGLLNGNSITVGSGGAVTNFTLAAGGTAVWQYTA-PETSPAIGNVGPTMGQPGNIV 543
BOHB        TDELQQLLDGNEITVNSNGAVDSFQLSANGVSVWQITE-EHASPLIGHVGPMMGKHGNTV 536
BREV        TDELQQLLDGNTITVNANGSANSPQLQANSVAVWQVTK-ESTSPLIGHVGPMIGKTGNTV 525
BF290       EDELSGLLDGQSITVDQNGSIQPFLLAPGEVSVWQYSNGQNVAPEIGQIGPPIGKPGDEV 531
KLEPN       PSLIG------NNSVSVANKRTTLTLMQNEAVVIRSQSDDAENPTVQSIN---------- 563
                . :        :    .      *  . . * :         *    :.

pT          TIDGRGFGSGKGTVYFGTTAVTGADIVAWEDTQIQVKIPAVPGGIYDIRVANAAGAASNI 602
pRB         TIDGRGFGSGKGTVYFGTTAVTGADIVAWEDTQIQVKIPAVPGGIYDIRVANAAGAASNI 602
A11         TIDGRGFGSGKGTVYFGTTAVTGADIVAWEDTQIQVKIPAVPGGIYDIRVANAAGAASNI 602
B382        TIDGR-ASARQGTVYFGTTAVTGADIVAWEDTQIQVKILRVPGGIYDIRVANAAGAASNI 600
B1011       TIDGRGFGSGKGTVYFGTTAVTGADIVAWEDTQIQVKIPAVPGGIYDIRVANAAGAASNI 602
B1018       TIDGRGFGSSKGTVYFGTTAVSGANITSWEDTQIKVKIPAVAGGIYNIKVANAAGTASNV 602
BC251       TIDGRGFGSSKGTVYFGTTAVSGADITSWEDTQIKVKIPAVAGGNYNIKVANAAGTASNV 602
BLICH       TIDGRGFGSAKGTVYFGTTAVTGSAITSWEDTQIKVTIPPVAGGDYAVKVA-ANGVNSNA 607
BCIR8       TIDGRGFGSTKGTVYFGTTAVTGAAITSWEDTQIKVTIPSVAAGNYAVKVA-ASGVNSNA 607
THETU       TIDGRGFGTTSGQVLFGSTAGT---IVSWDDTEVKVKVPSVTPGKYNISLKTSSGATSNT 599
BMACE       TIDGRGFGGTAGTVYFGTTAVTGSGIVSWEDTQIKAVIPKVAAGKTGVSVKTSSGTASNT 603
BOHB        TITGEGFGDNEGSVLFDS---DFSDVLSWSDTKIEVSVPDVTAGHYDISVVNAGDSQSPT 593
BREV        TVSGEGFGDKKGSVLFGS---TSAEIVSWSNTEIQVKVPNVTAGHYNLSVVNATNTKSPA 582
BF290       RIDGSGFGSSTGDVSFAG---STMNVLSWNDDTIIAELPEHNGGKNSVTVTTNSGESSNG 588
KLEPN       ------------------------------------------------------------

pT          YDNFEVLTGDQVTVRFVINNATTALGQNVFLTGNVSELGNWDP-NNAIGPMYNQVVYQYP 661
pRB         YDNFEVLTGDQVTVRFVINNATTALGQNVFLTGNVSELGNWDP-NNAIGPMYNQVVYQYP 661
A11         YDNFEVLTGDQVTVRFVINNATTALGQNVFLTGNVSELGNWDP-NNAIGPMYNQVVYQYP 661
B382        YDNFEVLTGDQVTVRFVINNATTALGQNVFLTGNVSELGNWDP-NNAIGPMYNQVVYQYP 659
B1011       YDNFEVLTGDQVTVRFVINNATTALGQNVFLTGNVSELGNWDP-NNAIGPMYNQVVYQYP 661
B1018       YDNFEVLSGDQVSVRFVVNNATTALGQNLYLTGNVSELGNWDP-AKAIGPMYNQVVYQYP 661
BC251       YDNFEVLSGDQVSVRFVVNNATTALGQNVYLTGSVSELGNWDP-AKAIGPMYNQVVYQYP 661
BLICH       YNDFTILSGDQVSVRFVINNATTALGENIYLTGNVSELGNWTTGAASIGPAFNQVIHAYP 667
BCIR8       YNNFTILTGDQVTVRFVVNNASTTLGQNLYLTGNVAELGNWSTGSTAIGPAFNQVIHQYP 667
THETU       YNNINILTGNQICVRFVVNNASTVYGENVYLTGNVAELGNWDT-SKAIGPMFNQVVYQYP 658
BMACE       FKSFNVLTGDQVTVRFLVNQANTNYGTNVYLVGNAAELGSWDP-NKAIGPMYNQVIAKYP 662
BOHB        YDKFEVLTGDQVSIRFAVNNATTSLGTNLYMVGNVNELGNWDP-DQAIGPMFNQVMYQYP 652
BREV        YEKFEVLSGNQVSVRFAVNNATTNSGTNVYIVGNVSELGNWDP-NKAIGPMFNQVMYKYP 641
BF290       YP-FELLTGLQTSVRFVVNQAETSVGENLYVVGDVPELGSWDP-DKAIGPMFNQVLYSYP 646
KLEPN       ---------------FTCNNGYTISGQSVYIIGNIPQLGGWDL-----TKAVKISPTQYP 603
                         *  *:. *  * .::: *.  :**.*       :     **

pT          TWYYDVSVPAGQTIEFKFLKKQ----GSTVTWEGGANRTFTTPTSG--TATDECELASLN 715
pRB         TWYYDVSVPAGQTIEFKFLKKQ----GSTVTWEGGCESHLSPPQPAGYWLTDECELASLN 717
A11         TWYYDVSVPAGQTIEFKFLKKQ----GSTVTWEGGANRTFTTPTSG---------TATMN 708
B382        TWYYDVSVPAGQTIEFKFLKKQ----GSTVTWEGGANRTFTTPTSG---------TATVN 706
B1011       TWYYDVSVPAGQTIEFKFLKKQ----GSTVTWEGGANRTFTTPTSG---------TATVN 708
B1018       NWYYDVSVPAGKTIEFKFLKKQ----GSTVTWEGGSNHTFTAPSSG---------TATIN 708
BC251       NWYYDVSVPAGKTIEFKFLKKQ----GSTVTWEGGSNHTFTAPSSG---------TATIN 708
BLICH       TWYYDVSVPAGKQLEFKFFKKN----GATITWEGGSNHTFTTPTSG---------TATVT 714
BCIR8       TWYYDVSVPAGKQLEFKFFKKN----GSTITWESGSNHTFTTPASG---------TATVT 714
THETU       TWYYDVSVPAGTTIQFKFIKKN----GNTITWEGGSNHTYTVPSSS---------TGTVI 705
BMACE       SWYYDVSVPAGTKLDFKFIKKG----GGTVTWEGGGNHTYTTPASG---------VGTVT 709
BOHB        TWYYDISVPAEENLEYKFIKKDS---SGNVVWESGNNHTYTTPATG---------TDTVL 700
BREV        TWYYDISVPAGKNLEYKYIKKDH---NGNVTWQSGNNRTYTSPATG---------TDTVI 689
BF290       TWYYDVSVPANQDIEYKYIMKDQ---NGNVSWESGNNHIYRTPENS---------TGIVE 694
KLEPN       QWSASLELPSDLNVEWKCVKRNETNPTANVEWQSGANNQFNSNDTQ----------TTN 652
              *  .:.:*:   :::* . :       .: *:.* :

pT          RHLQGKQAAPGRGSGPLVTLCGGKVL 741
pRB         RHLQGKQAAPGRG---LL-------- 732
A11         VNWQP--------------------- 713
B382        VNWQP--------------------- 711
B1011       VNWQP--------------------- 713
B1018       VNWQP--------------------- 713
BC251       VNWQP--------------------- 713
BLICH       INWQ---------------------- 718
BCIR8       VNWQ---------------------- 718
THETU       VNWQQ--------------------- 710
BMACE       VDWQN--------------------- 714
BOHB        VDWQ---------------------- 704
BREV        SNW----------------------- 692
BF290       VNFNQ--------------------- 699
KLEPN       GSF----------------------- 655
```

Figure 10 (cont'd)

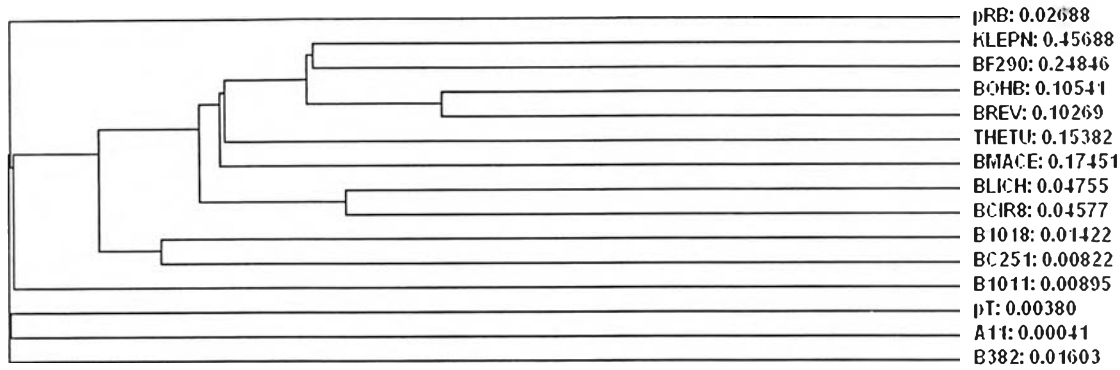


Figure 11 Phylogenetic tree of the sequence alignment of various published CGTase sequences. The tree was built by the neighbor joining method, implemented in the CLUSTALW program (**79**). The accession number and abbreviation of each CGTase were listed in Table 4

Table 4 List of CGTases used in the sequence alignment and phylogenic tree construction.

Abbreviation	Accession number	Source	Main Product	Reference
KLEPN	P08704	*K. pneumoniae* strain M5a1	α	(**39**)
BMACE	P04380	*B. macerans* strain NRRL B388	α	(**80**)
BLICH	P14014	*B. licheniformis*	α / β	(**81**)
B1011	P05618	Alkalophilic *B.* sp. strain 1011	β	(**37**)
BCIR8	AA4840	*B. circulans* strain 8	β	(**82**)
B1018	P17692	*Bacillus* sp. strain B1018	β	(**83**)
B382	P09121	Alkalophilic *B.* sp. strain 38.2	β	(**38**)
BC251	P43379	*B. circulans* strain 251	β	(**84**)
BOHB	BAA14289	*B. ohbensis* (strain C-1400)	β (no α)	(**85**)
THETU	P26827	*T. thermosulfurigenes* EM1	β /α	(**86**)
BREV	AAB65420	*Brevibacillus brevis* strain CD162	γ/ β	(**87**)
BF290	A18991	*B. firmus/lentus* strain 290-3	γ/ β	(**88**)
A11	AAG31622	*Paenibacillus sp.* A11	β	(**69**)

3.3 Expression of CGTases in the original cell isolates and recombinant cells

In preliminary experiments, the wild types and the transformants were checked for the optimum conditions for culturing such as type of medium and incubation time. Two kinds of medium, Horikoshi pH 10.0 and LB medium pH 7.0 were chosen. The results showed that the wild types preferred the Horikoshi medium but the transformants could not grow at alkaline pH of Horikoshi medium.

The cells from RB01, pRB, T16 and pT were taken from the stocks and prepared for cultivation by inocutation in an appropriate medium and follwed steps of preparation as described in sections 2.5, 2.6.1 and 2.10. The cultivated media from each strain was collected for the determination of the absorbance at 660 nm and enzyme assayed at time interval of 6, 12, 18, 24, 36, 48 and 72 hours. For the enzyme assay, cells were removed from the media by centrifugation (3,000g) at 4°C for 30 minutes, and the supernatant fraction containing CGTase was collected and dextrinizing activity was assayed determined as described in the section 2.9.1.

Figure 12 (a, b and c) showed that rapid growth was observed in both of the original cells and the transformant from 12 hour of incubation. However, the transformant showed the highest dextrinizing activity around 18-24 hours incubation, while the wild type needed the longer incubation time of 60-72 hours to reach the highest dextrinizing activity. The specific activity of the transformant pT was about 3 times higher than that of the wild type.

Therefore, RB01 and T16 were cultivated in Horikoshi medium containing 1.0% soluble starch, at pH 10.0, 40°C, 250 rpm for 72 hours which was optimum condition determined by Tesana, 2001 and Pranomitra, 2001. While, the transformants, pRB and pT were cultivated in LB medium containing 1.0% soluble starch, at pH 7.0, 37°C, 250 rpm for 24 hours.

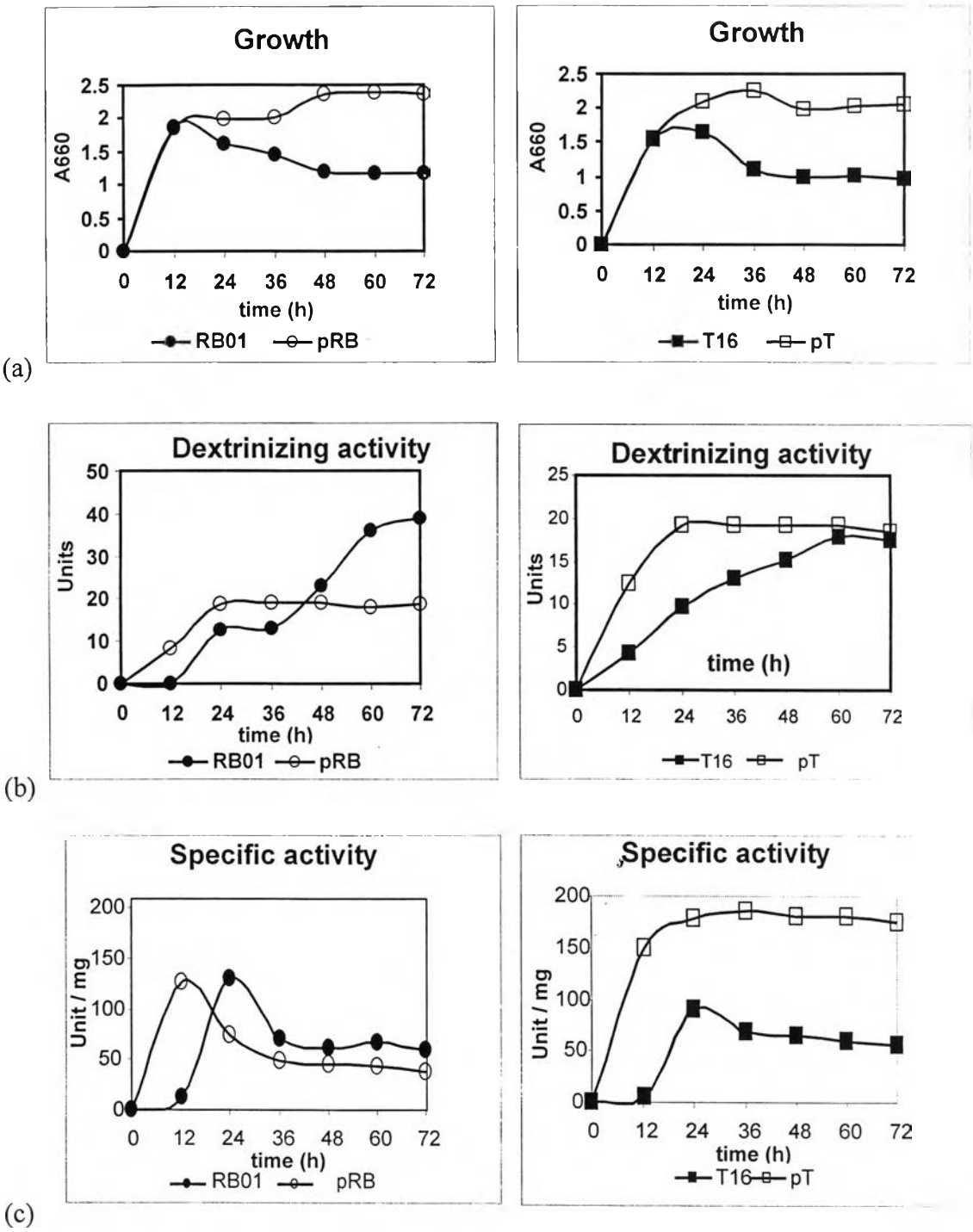


Figure 12 Growth and CGTase activity profiles of RB01, pRB, T16 and pT.

(a) Growth profile (b) total dextrinizing activity (c) specific activity.

RB01 and T16 were cultivated in Horikoshi medium containing 1.0% soluble starch, at pH 10.0, 40°C, 250 rpm for 72 hours. While, pRB and pT were cultivated in LB medium containing 1.0% soluble starch and 100 μg/ml ampicillin , at pH 7.0, 37°C, 250 rpm for 24 hours.

3.4 Purification of CGTases

Purification of CGTases were carried out for enzyme from both the isolates and recombinant cells of RB01 and T16.

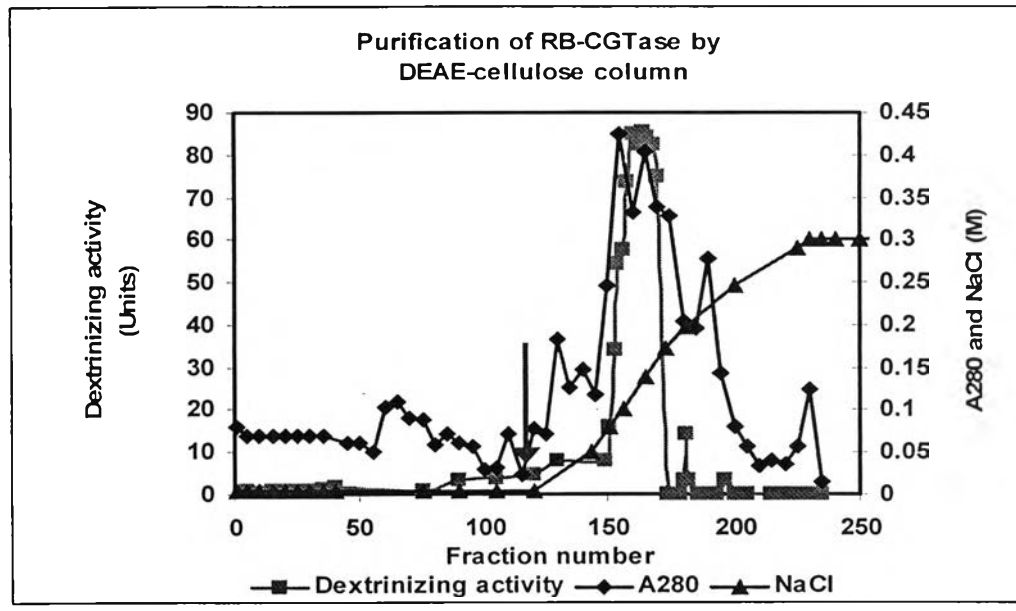
3.4.1 Purification of CGTase from RB01 and pRB

The wild type, RB01 was cultivated in 1,000 ml of Horikoshi and 1% soluble potato starch at 40°C with 250 rpm shaking for 72 hours. The recombinant, pRB, was cultivated in 1,200 ml of LB broth containing 100 mg/ml of ampicillin and 1% soluble potato starch at 37°C with 250 rpm shaking for 24 hours. After cultivation, bacterial cell mass was removed by centrifugation at 4,500 rpm for 15 minutes at 4°C. Culture broth with crude enzyme was collected and kept at 4°C for activity assay and determination of protein content before the next step of purification. Crude enzyme was purified by starch adsorption as described in method section 2.11.1, followed by DEAE-cellulose chromatography column as described in method section 2.11.2.

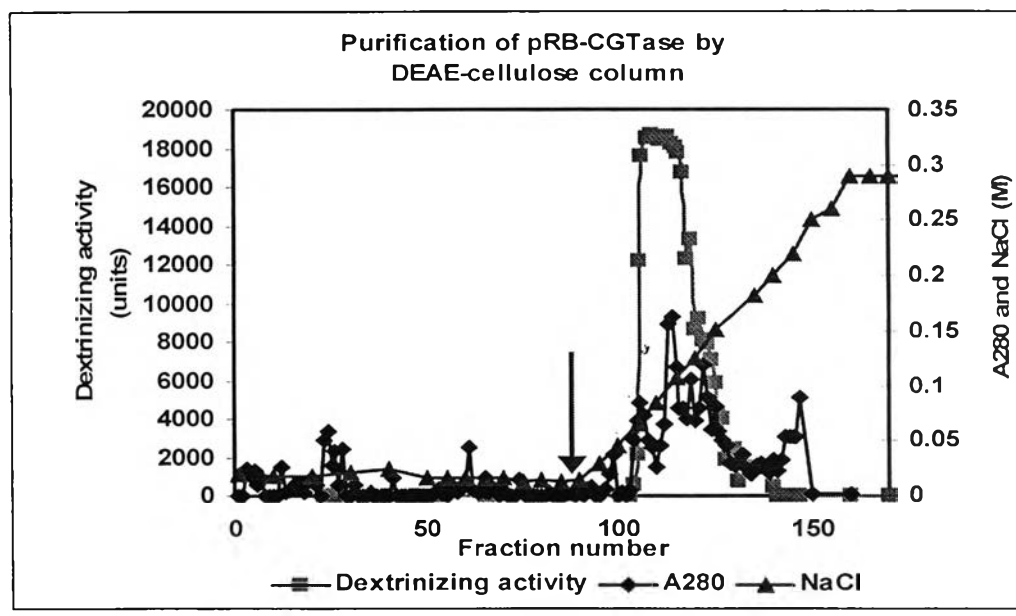
The crude enzyme contained the total protein of 388.9 mg with 59,500 units of CGTase in the total volume of 1,000 ml for RB01 and 293.9 mg with 210,200 units of CGTase in the total volume of 1,150 ml for pRB, respectively. Thus, the specific activity of RB01 and pRB were 153 and 715 units/mg, respectively. The crude enzyme solution was further purified by starch adsorption. In this step, the purification fold and recovery of CGTase obtained were 29 folds and 72% yield for RB01, 3 folds and 22% yield for pRB. The specific activities of the enzymes from this step were 4,435 units/mg for RB01 and 2,283 units/mg for pRB.

For RB01 and pRB the enzymes from the starch adsorption step were concentrated with the ultrafiltration (Viva-Flow 50) and dialyzed against 10 mM Tris-HCl buffer, pH 8.5 (TB2). Each enzyme solution was applied onto DEAE-cellulose column as described in the section 2.11.2. The chromatographic profile of RB01 and

pRB were shown in Figures 13 (a) and (b). The unbound proteins were eluted from the column with TB2 buffer. The bound proteins were eluted with linear salt gradient of 0 to 0.3 M sodium chloride in TB2 buffer. Both enzymes were eluted at approximately 0.1 M NaCl as indicated in the profile. The fractions with CGTase activity were pooled, concentrated by aquasorb to reduce enzyme volumes and dialyzed against TB2 buffer. The proteins remained in this step were 5.59 mg with 30,770 units of enzyme and specific activity of 5,504 units/mg protein for RB01 and 0.8 mg with 14,688 units of enzyme and specific activity of 18,360 units/mg protein for pRB (Table 5).



(a)



(b)

Figure 13 DEAE-cellulose column profiles of CGTases from RB01and pRB. The column size was 1.5x28 cm. The enzyme solution was applied to the DEAE-cellulose column and washed with 10 mM TB2 buffer, pH 8.5, until the A_{280} decreased to the baseline. Elution of bound proteins was performed by 0-0.3 M NaCl in the same buffer at the flow rate of 45 ml/h. Fractions of 4 ml were collected. The arrow indicates where the gradient started. The fractions of high protein peak and high CGTase activity were pooled.

(a) RB01 (b) pRB

Table 5 Purification of CGTase from *Paenibacillus* sp. strain RB01 and the transformant, pRB.

Step	Volume (ml)		Total activity* (Units)		Total protein (mg)		Specific activity (U/mg)		Purification Fold		Yield (%)	
	RB01	pRB	RB01	pRB	RB01	pRB	RB01	pRB	RB01	pRB	RB01	pRB
Crude	1000	1150	59500	210200	388.9	293.9	153	715	1	1	100	100
Starch adsorption	85	250	42840	47250	9.66	20.7	4435	2283	29	3	72	22
DEAE-cellulose	362	48	30770	14688	5.59	0.8	5504	18360	36	26	52	7

* Dextrinizing activity

3.4.2 Purification of CGTase from T16 and pT

The wild type T16 was cultivated in 1,600 ml of Horikoshi and 1% soluble potato starch at 40°C, shaking 250 rpm for 72 hours. The recombinant, pT was cultivated in 1,200 ml of LB broth containing 100 mg/ml of ampicillin and 1% soluble potato starch at 37^0C for 24 hours. After cultivation, bacterial cell mass was removed by centrifugation at 4,500 rpm for 15 minutes at 4^0C. Culture broth with crude enzyme was collected and kept at 4^0C for activity assay and determination of protein content before the next step of purification. Crude enzyme was purified by starch adsorption as described in method section 2.11.1, followed by phenyl sepharose chromatography column as described in method section 2.11.3.

The crude enzyme contained a total protein of 465.6 mg with 44,750 units of CGTase in the total volume of 1,600 ml for T16 and 188.71 mg with 181,900 units of CGTase in the total volume of 1,130 ml for pT, respectively. Thus, the specific activities of T16 and transformant, pT were 96 and 964 units/mg, respectively.

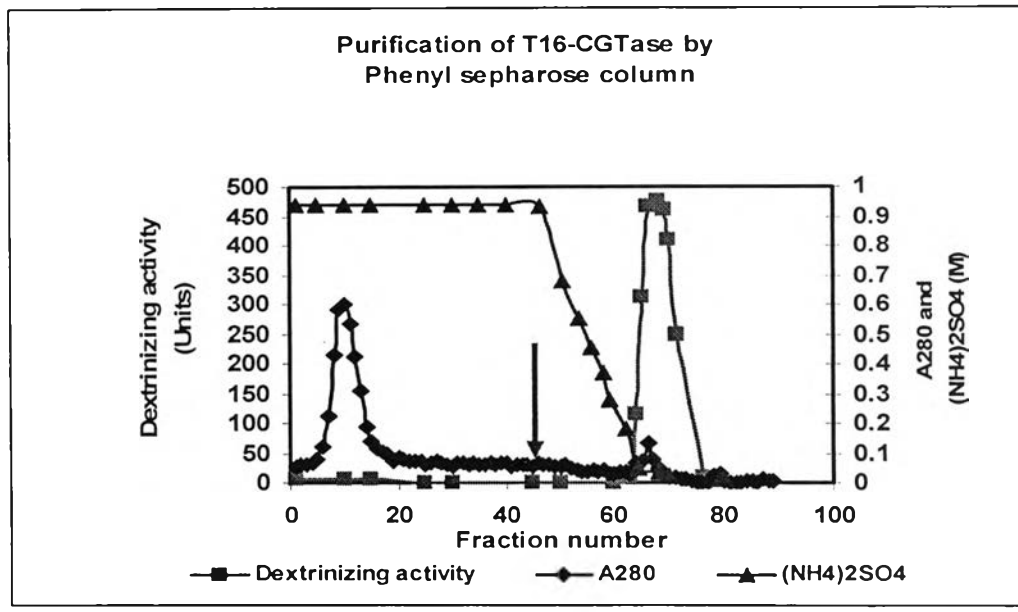
The crude enzyme solution was further purified by starch adsorption. The purification fold and recovery of CGTase obtained were 32 folds and 61% yield for T16 and 33 folds and 19% yield for pT, respectively. The specific activities of the enzymes from this step were 3,105 units/mg and 31,818 units/mg for pT.

The enzyme solutions from starch adsorption of T16 and pT were applied onto a phenyl sepharose column as described in the section 2.11.3. The chromatographic profiles were shown in Figure 14 (a) and (b). The unbound proteins were eluted from the column with the TB2 buffer containing 1 M ammonium sulfate. The bound proteins were eluted with decreasing concentration of ammonium sulfate linear gradient from 1 M to 0 M in buffer TB2. The enzyme was eluted at 0.05 M ammonium sulfate as indicated in the profiles. The fraction with CGTase activity were pooled, concentrated by aquasorb

to reduce enzyme volumes and dialyzed against buffer TB2. The proteins remained from this step were 0.98 mg with 9,486 units of enzyme and specific activity of 9,680 units/mg protein for T16 and 0.9 mg with 80,700 units of enzyme and specific activity of 89,667 units/mg protein for pT, respectively (Table 6).

The dextrinizing activity and specific activity of CGTase from wild-types, RB01 and T16 and their transformants, pRB and pT, respectively were compared in Table 7. The transformants produced about 3 times higher CGTase in pRB and 10 times higher enzyme in pT comparing with their repective wild type.

(a)



(b)

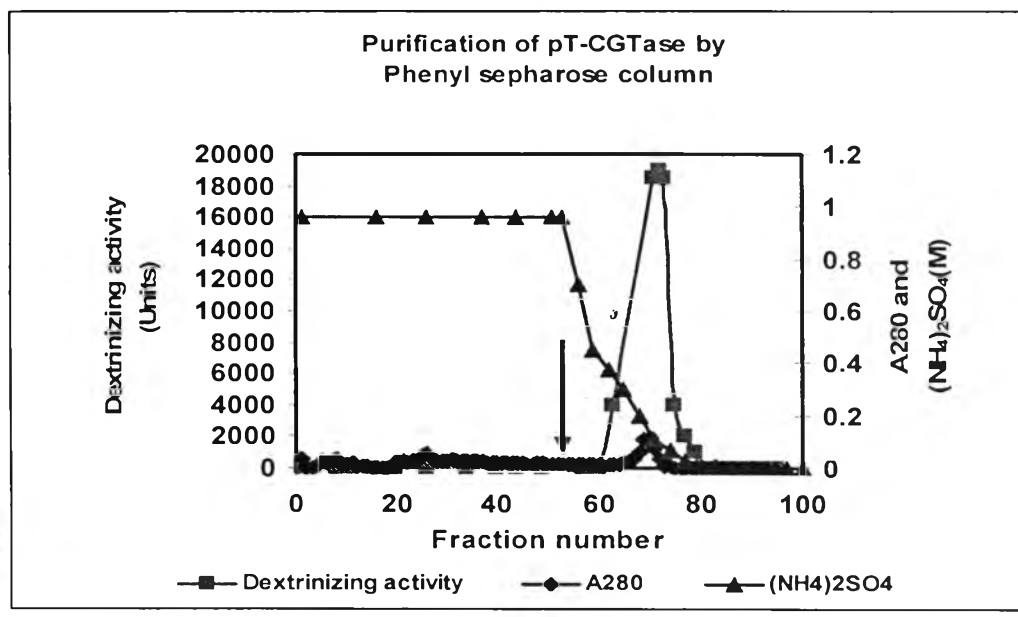


Figure 14 Phenyl sepharose column profiles of CGTases from T16 and pT. Column size was 1.3x10 cm, elution was by 1 - 0 M $(NH_4)_2SO_4$ in TB2 buffer (10 mM Tris buffer, pH 8.5). Fractions of 2 ml were collected. The enzyme solution was applied to phenyl-Sepharose CL-4B column and washed with TB2 buffer containing 1 M ammonium sulfate until A_{280} decreased to baseline. Elution of bound proteins was then performed by a decreasing linear gradient of ammonium sulfate from 1 to 0 M at the flow rate of 20 ml/hour. The arrow indicates where gradient started. The fractions of high protein peak and high CGTase activity were pooled.

(a) T16 (b) pT

Table 6 Purification of CGTase from *Paenibacillus* sp. strain T16 and the transformant, pT.

Step	Volume (ml)		Total activity* (Units)		Total protein (mg)		Specific activity (U/mg)		Purification Fold		Yield (%)	
	T16	pT	T16	pT	T16	pT	T16	pT	T16	pT	T16	pT
Crude	1600	1130	44750	181900	465.6	188.71	96	964	1	1	100	100
Starch adsorption	400	250	27320	35000	8.8	1.1	3105	31818	32	33	61	19
Phenyl Sepharose	21	23	9486	80700	0.98	0.9	9680	89667	101	93	21	44

* Dextrinizing activity

Table 7 Comparison the dextrinizing activity and specific activity between wild-type and transformant CGTase

Fraction	Dextrinizing activity (U/ml)				Specific activity (U/mg)			
	RB01	pRB	T16	pT	RB01	pRB	T16	pT
Crude	60	183	28	161	153	715	96	964
Starch Adsorption	504	189	68	140	4435	2283	3105	31818
Purified column*	85	306	452	3509	5504	18360	9680	89667

* DEAE-cellulose column for purification of RB01 and pRB,

* Phenyl sepharose column for purification of T16 and pT

3.5 Characterization of purified CGTases

3.5.1 Determination of enzyme purity and protein pattern on non-denaturing and SDS-PAGE

The purified enzyme from each source was analyzed for purity and protein pattern by non-denaturing polyacrylamide gel electrophoresis. The activity stain was performed in comparison with protein stain. The result in Figure 15 showed many protein bands in the crude enzyme from pRB but only 2-3 band with one major band in the purified form. Iodine stain showed a positive clear band in all samples. SDS-PAGE of purified CGTase from RB01 and pRB showed only a single band in SDS-PAGE as presented in Figure 16. From the mobility on SDS-PAGE, the molecular weight of CGTase from pRB was estimated to be 65 kDa (Figure 19 (a)).

Similar pattern of native and SDS-PAGE analysis were obtained with pT (Figures 17, 18), the molecular weight of pT on SDS-PAGE was estimated to be 77 kDa (Figure 19 (b)).

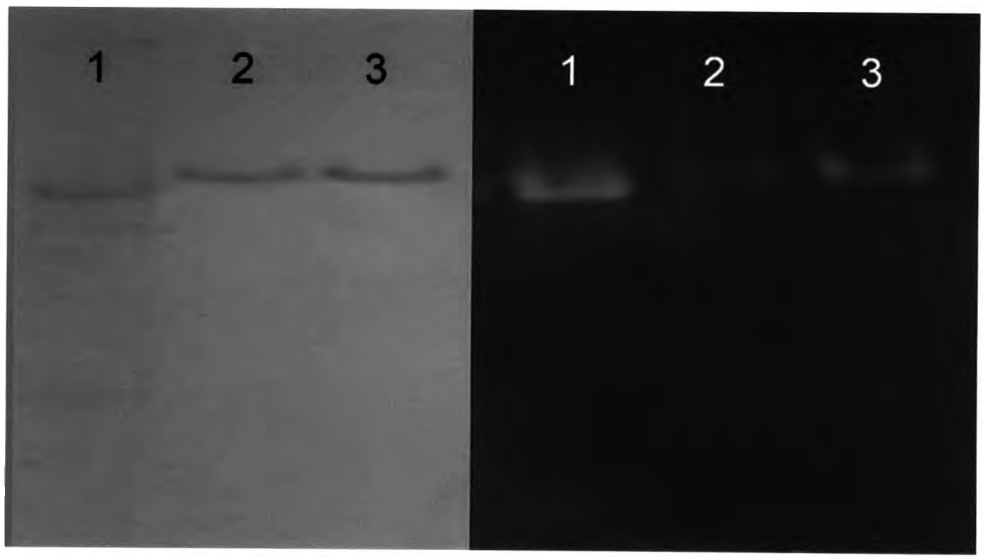


Figure 15 Non-denaturing PAGE analysis of pRB-CGTase from different purification steps.

Lane 1 Crude, lane 2 Starch adsorbed, lane 3 DEAE cellulose column

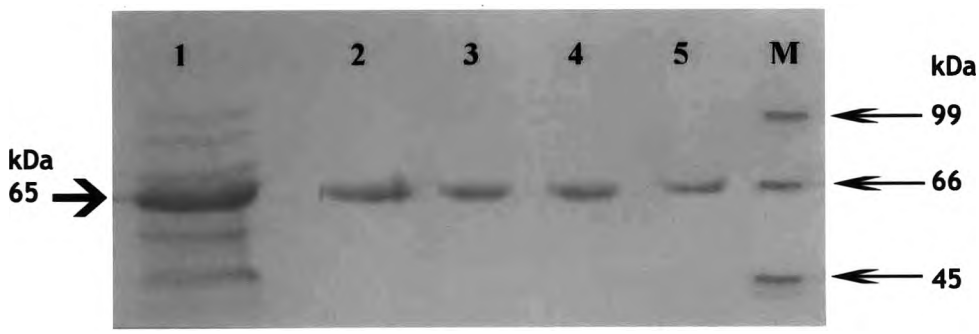


Figure 16 SDS-PAGE analysis of pRB-CGTase from different purification steps.

Lane 1 Crude , Lane 2 Starch adsorbed, Lane 3 DEAE cellulose column, Lane 4 Starch adsorbed from Wild type, Lane 5 BSA, M = Low Molecular weight markers

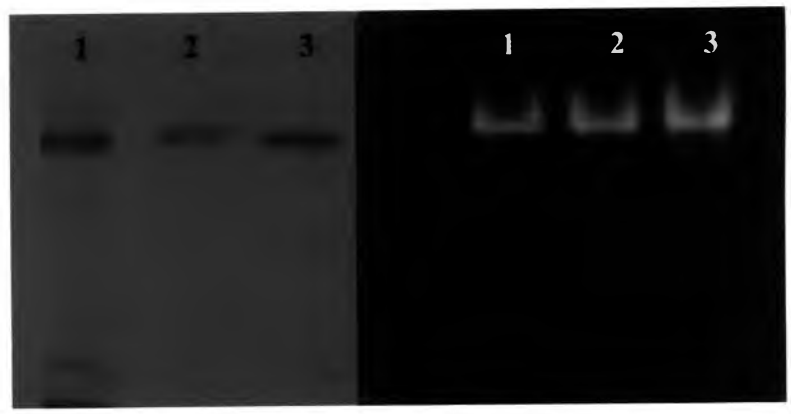


Figure 17. Non-denaturing PAGE analysis of pT-CGTase from different purification steps. (lane 1-3) Lane 1 Crude, Lane 2 Starch adsorbed, Lane 3 DEAE cellulose column

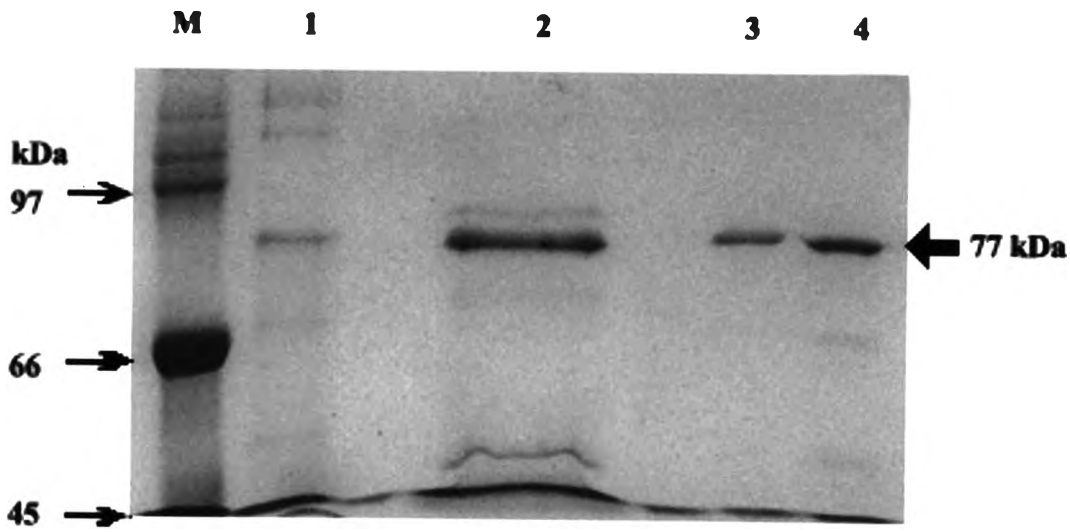
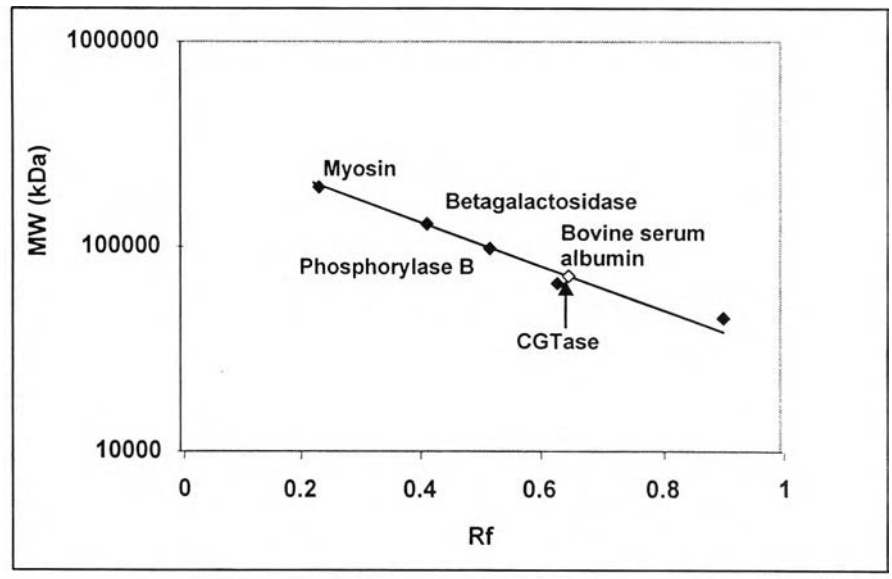
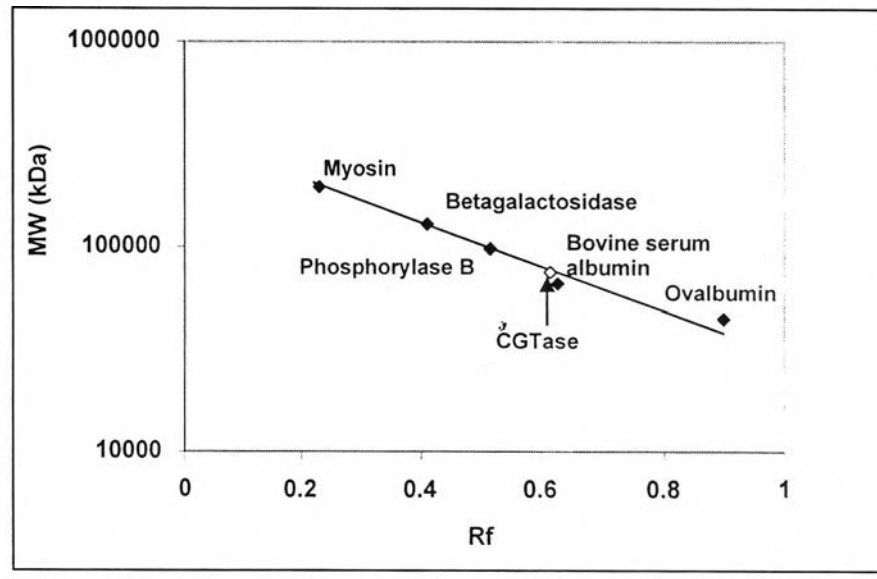


Figure 18 SDS-PAGE analysis of pT-CGTase from different purification steps. (lane 2-4) Lane 1 Crude from wild type , Lane 2 Crude from pT, Lane 3 Starch adsorbed. Lane 4 Phenyl Sepharose column



(a)



(b)

Figure 19 Molecular weight calibration curve of standard proteins by SDS-PAGE

Myosin	205	kDa
β-galactosidase	116	kDa
Phosphorylase B	97	kDa
Bovine serum albumin	66	kDa
Ovalbumin	45	kDa

Arrow indicated the molecular weight of CGTase from this study were in range of 65 kDa from RB01 (a) and 77 kDa from T16 (b)

3.5.2 Determination of pI

Purified CGTases were analyzed for their isoelectric points by separation on IEF gel electrophoresis, comparing to standard pI markers. Ampholine pH range 3-10 was used and relative mobility against pI was plotted. Sample from pRB and pT showed one major band at pI 5.85. pRB also showed 2 faint bands at pI 5.74, 5.34. Sample from wild type RB01 showed very faint bands at 5.74, 5.64, 5.34 and T16 at 5.74, 5.54, 5.34.

3.5.3 Optimum conditions for enzyme activity

3.5.3.1 Effect of pH on CGTase activity

The effect of pHs on the enzyme activity was studied at various pH's as mentioned in section 2.12.3.1, taking the pH with highest enzyme activity as 100%. The results showed that the wild type and the transformant of both RB01 and T16 had the same pattern of pH-activity profiles of dextrinizing activity (Figures 22, 23) and cyclization activity (Figures 24, 25). The optimum pH for dextrinizing activity and cyclization was 5.5-9.5 and 6.5-7.0, respectively. The enzyme lost activities at extreme acidic and alkaline pH's.

3.5.3.2 Effect of temperature on the enzyme activity

The effect of temperature on the purified enzymes were investigated by incubating the reaction mixtures at various temperatures as mentioned in section 2.12.3.2 and determined for both dextrinizing and cyclization activities. The optimum temperature for dextrinizing activity of CGTase from RB01 and T16 were 70°C, and 60°C, respectively. However, pRB and pT showed broader optimum temperatures. At 50-70°C, pRB still exhibited dextrinizing activity at higher than 90% while pT retained activity at greater than 90% in the temperature range of 40-75°C (Figures 26, 27). The optimum temperatures for cyclization activity of all strains were quite similar in the range of 50-70°C, although the transformants showed slightly higher activities.

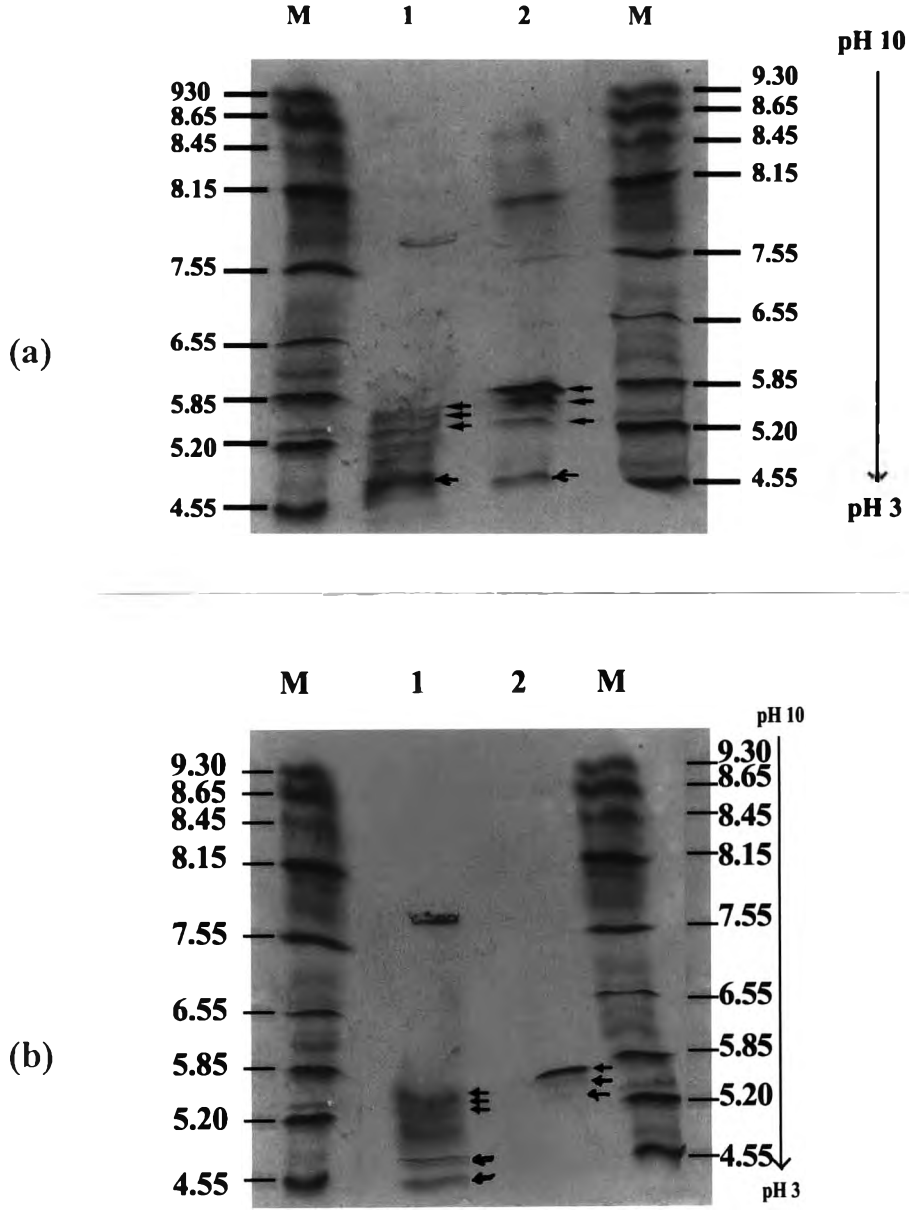
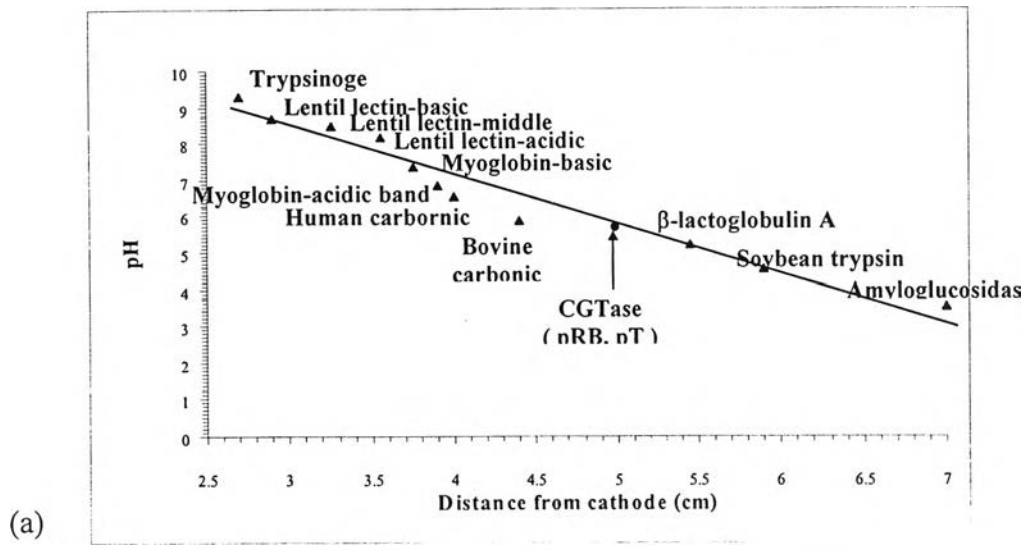


Figure 20 Isoelectric focusing gel with ampholyte solution (pH 3.0-10.0) of purified CGTase

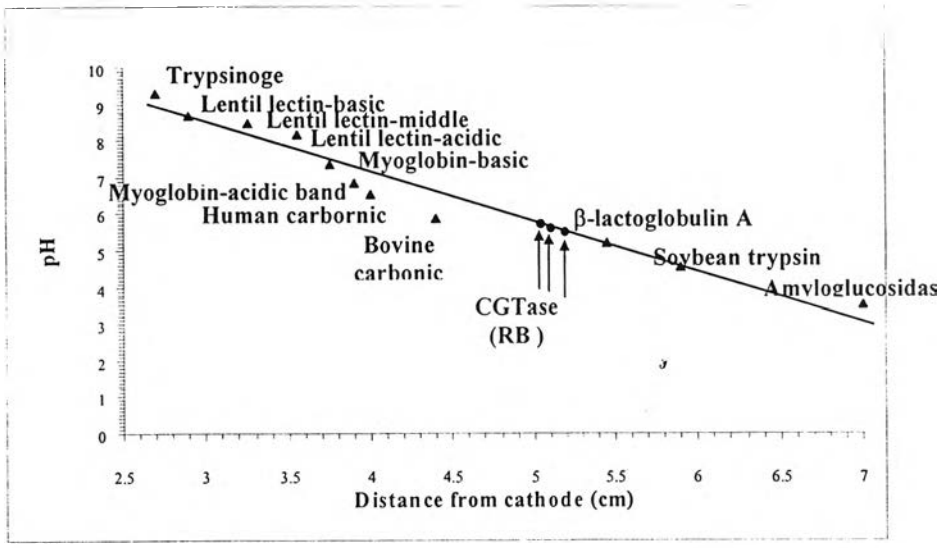
(a) Lane 1 : Purified CGTase from RB01
Lane 2 : Purified CGTase from pRB

(b) Lane 1 : Purified CGTase from T16
Lane 2 : Purified CGTase from pT

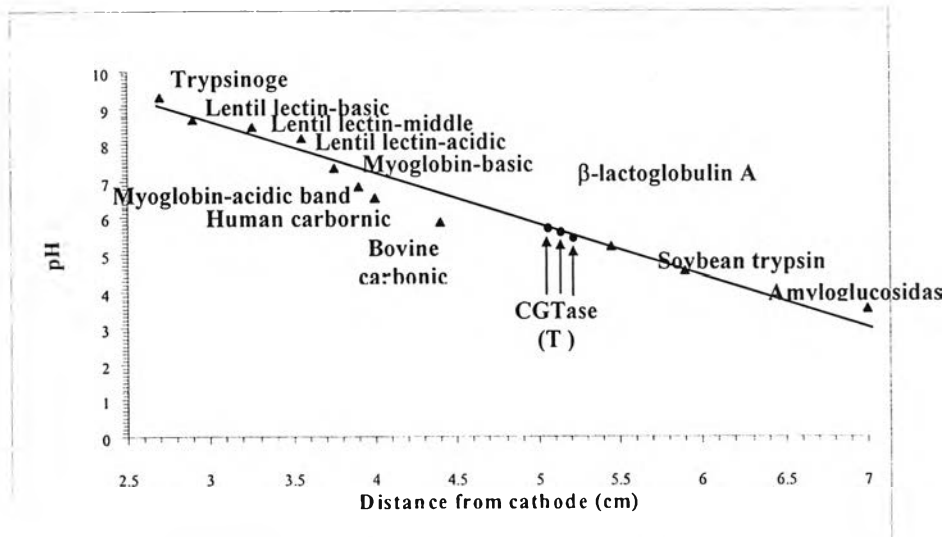
Lane M Standard pI markers; Amyloglucosidase (3.50), Soybean trypsin inhibitor (4.55), β-lactoglobulin (5.20), bovine carbonic anhydrase B (5.85), human carbonic anhydrase B (6.55), myogloblin-acidic band (6.85), myoglobin-basic band (7.35), lentil lectin-acidic band (8.15), lentil lectin-middle band (8.45), lentil lectin-basic band (8.65), trypsinogen (9.30).



(a)



(b)



(c)

Figure 21 Standard curve of pI determination

▲ = Standard proteins ● = CGTase (pRB, pT (a), RB01 (b), T16 (c))

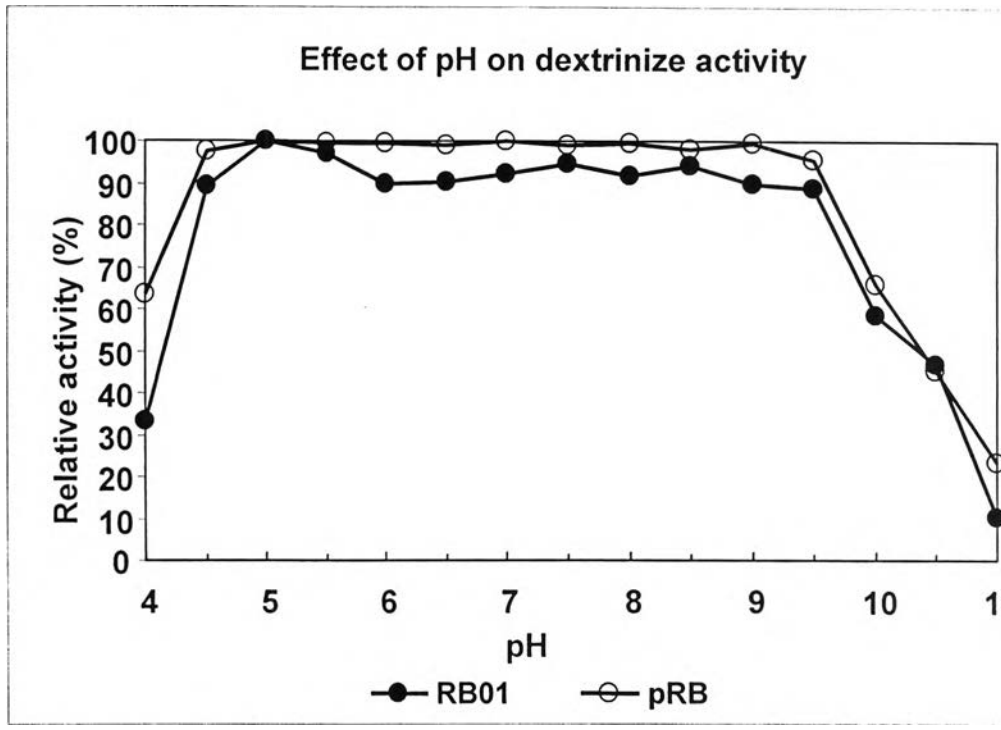


Figure 22 Effect of pH on dextrinizing activity of CGTase from RB01 and pRB at 40°C

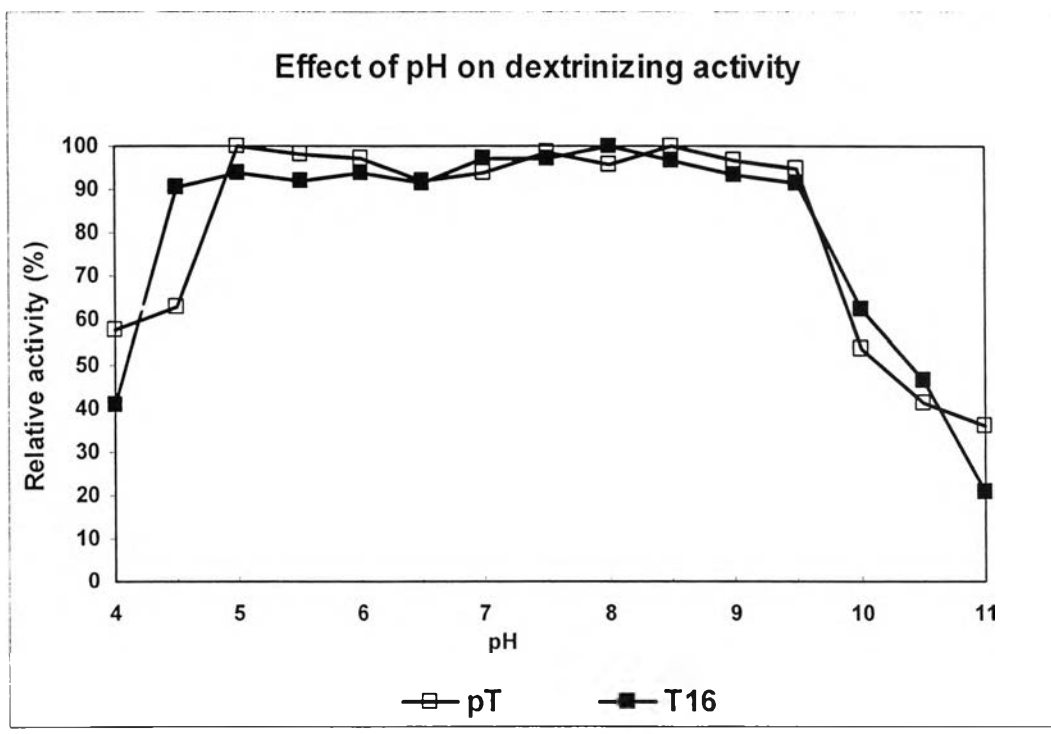


Figure 23 Effect of pH on dextrinizing activity of CGTase from T16 and pT at 40°C

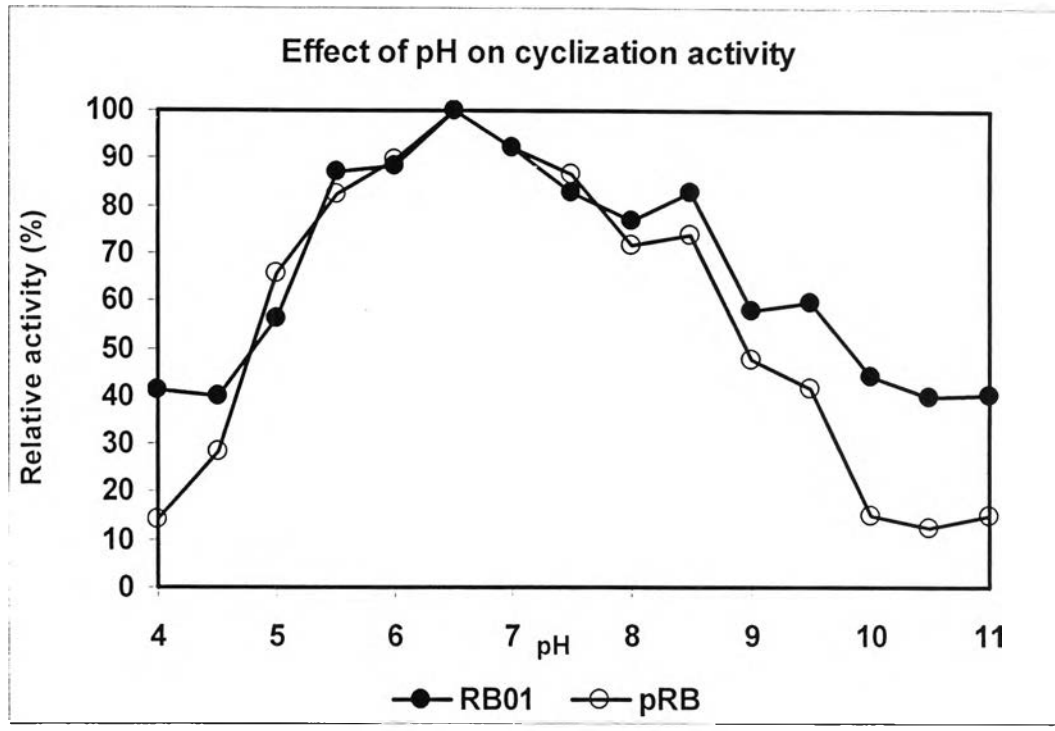


Figure 24 Effect of pH on cyclization activity of CGTase from RB and pRB at 60°C

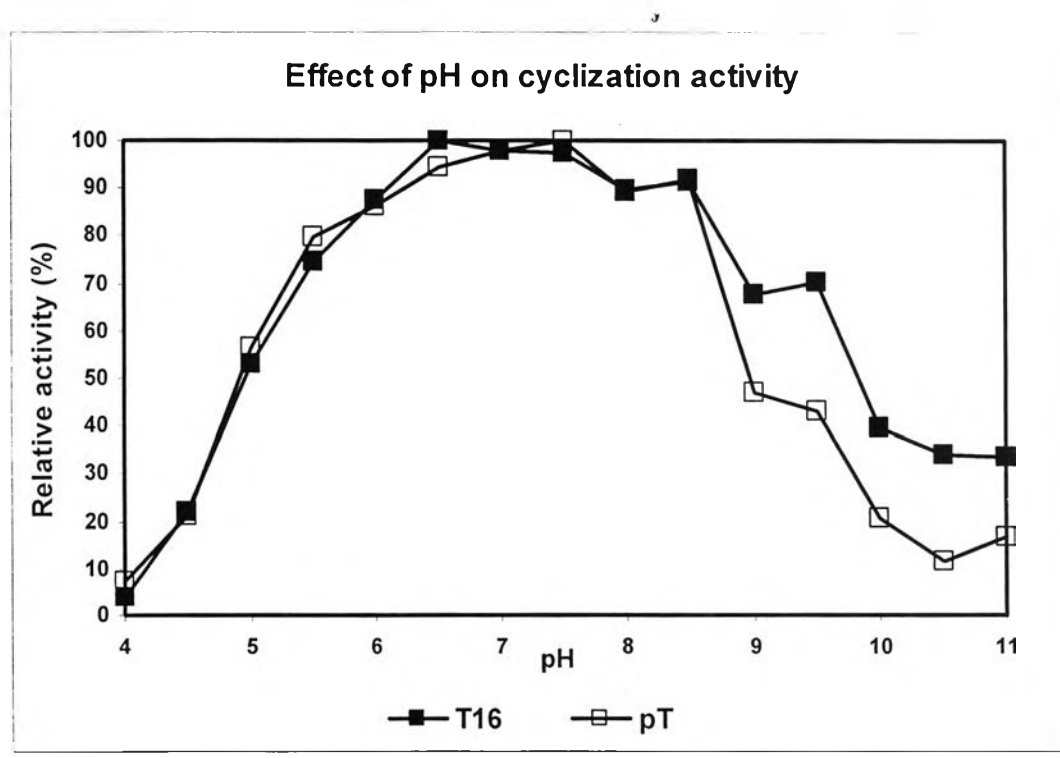


Figure 25 Effect of pH on cyclization activity of CGTase from T16 and pT at 60°C

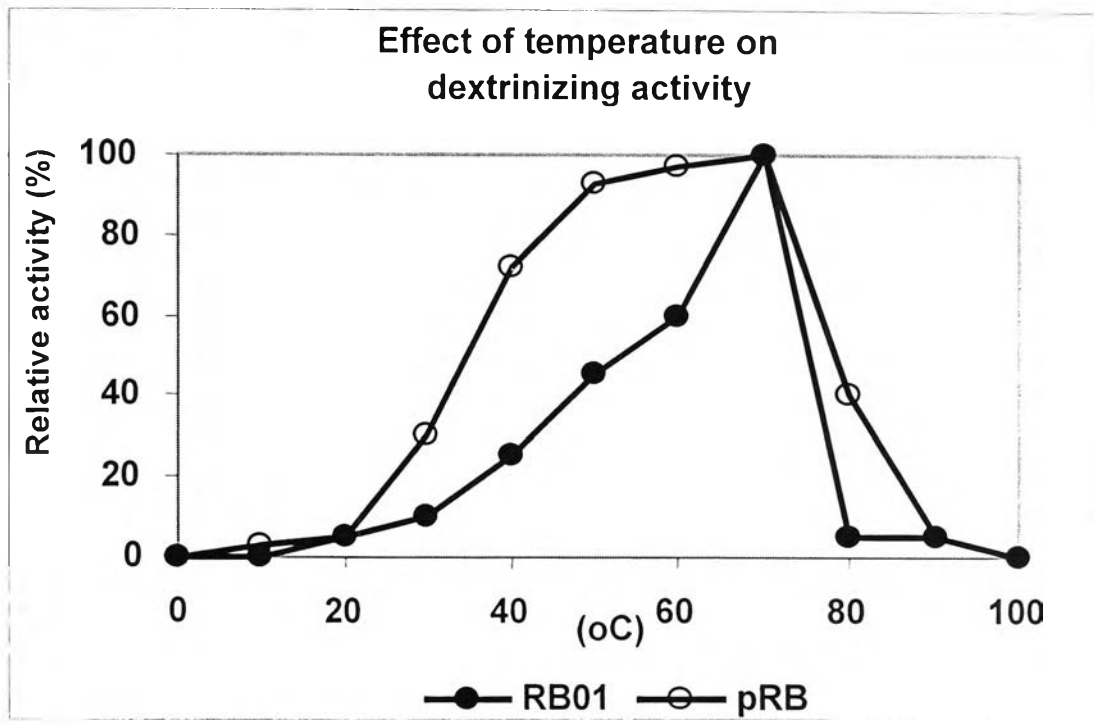


Figure 26 Effect of temperature on dextrinizing activity of CGTase from RB01 and pRB at pH 6.0

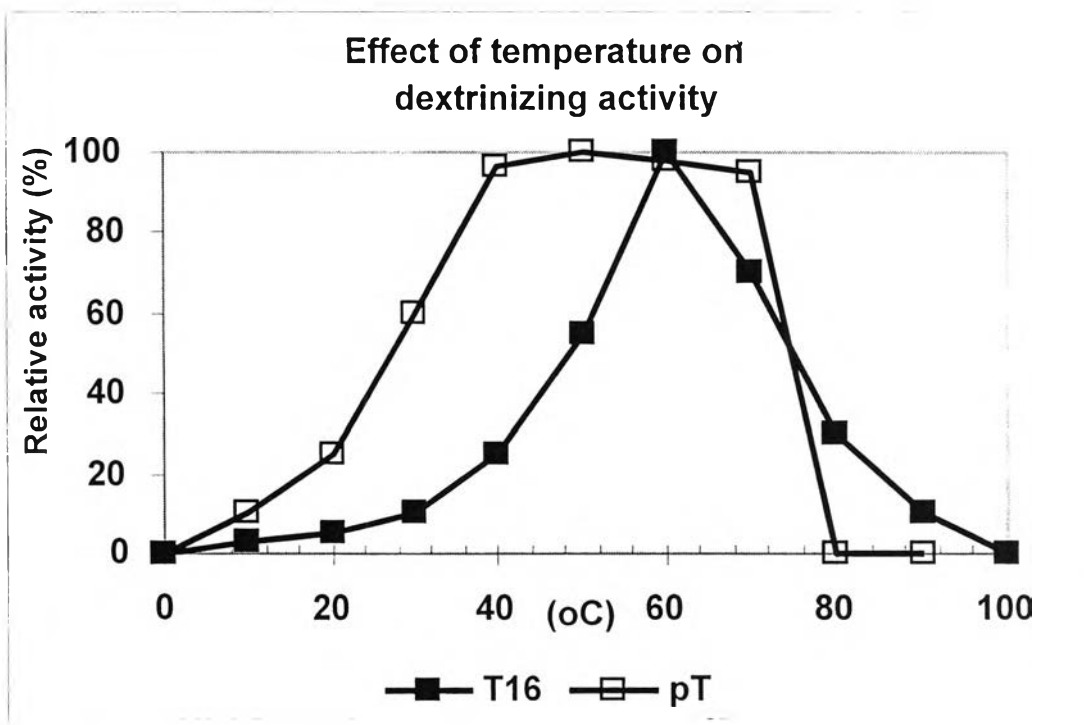


Figure 27 Effect of temperature on dextrinizing activity of CGTase from T16 and pT at pH 6.0

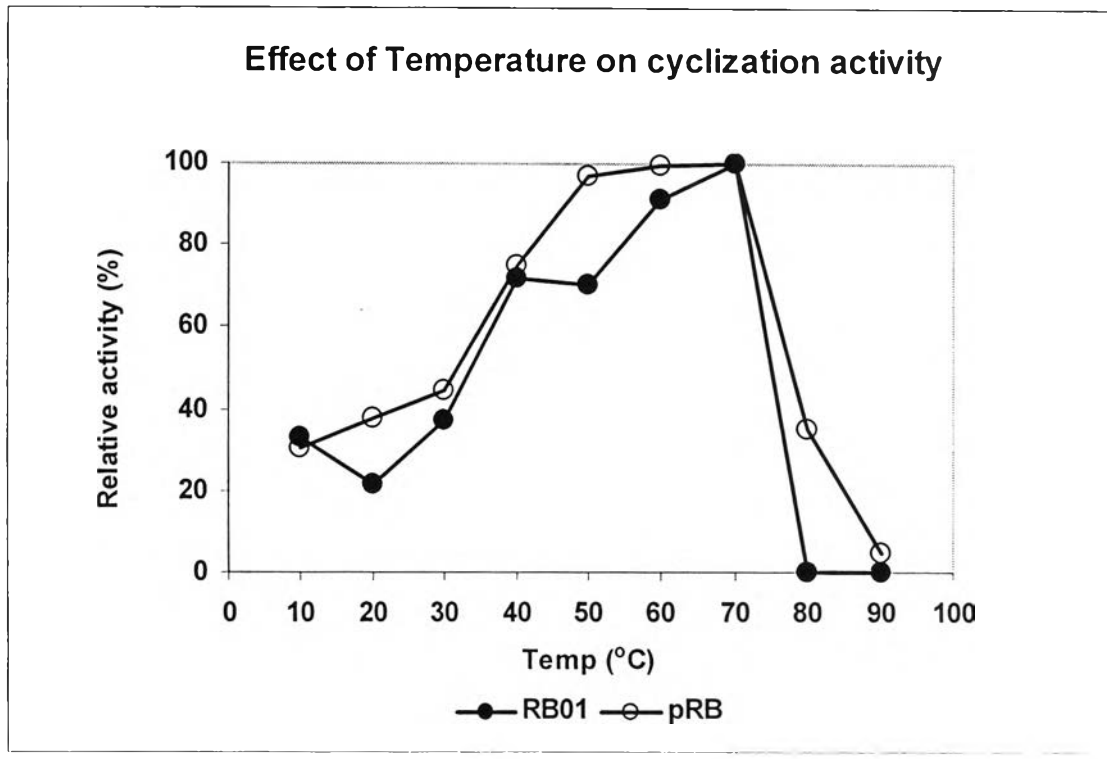


Figure 28 Effect of temperature on cyclization activity of CGTase from RB01 and pRB at 60°C

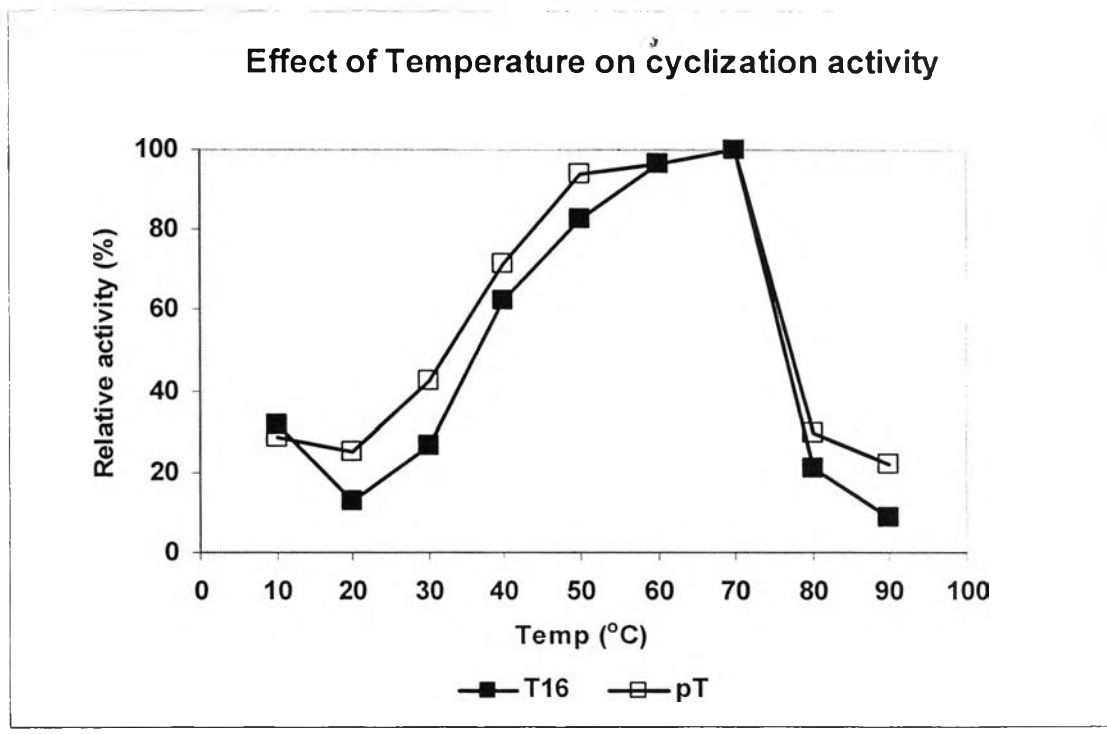


Figure 29 Effect of temperature on cyclization activity of CGTase from T16 and pT at 60°C

3.5.4 Stability of purified CGTase

3.5.4.1 pH stability

The effect of pH on enzyme stability was investigated on the cyclization activity by incubating 20 units of enzyme for one hour at 55 °C for all four enzymes which were their optimum temperatures in buffers of varying pH prior to determination of cyclization activity under standard assay conditions as described in section 2.9.2. As shown in Figures 30-33, the cyclization activity of all strains remained stable between pH 6 and 9 at 60°C. At pH 5, the cyclization activity of all strains except pT rapidly lost activity after preincubation for 20 minutes. The cyclization activity of CGTase from pT at pH 5 slowly declined and retained 60 % activity after preincubation at 55°C for one hour. The cyclization activities of all strains retained up to 60% relative activity after preincubation at pH 6 to 10, at 55°C for 40 minutes. However, CGTase from T16 preincubated at pH 6.0 to 10, completely lost the cyclization activity after 40 minutes, excepted for pH 7. The cyclization activity of CGTase from pT was quite stable at all pH's up to 40 minutes after which its activity slowly declined and retained around 50% after preincubation for one hour.

3.5.4.2 Temperature stability

Each purified CGTase (20 units of enzyme) was preincubated at the designated temperature at pH 7.0 up to one hour. Samples were taken at 5 minutes intervals and assayed for cyclization activity as described in section 2.9.2. For RB01, the temperature stability pattern of the wild type and recombinant were similar. CGTases from RB01 and pRB retained 80% or more of their activities at 40-60°C up to one hour. At 65°C, they were relatively stable up to 30 minutes (Figures 34, 35). At 70°C, their activity declined rapidly from the start of incubation to a minimum around 20 minutes. The presence of

2% starch at 70°C stabilized the enzymes which retained activity at 40% for RB and 60% for pRB after 60 minutes.

Cyclization activity of pT (Figure 36) seemed to be more stable, retaining its activity up to 80% after 60 minutes while activity of T16 (Figure 37) dropped to 20% at 65°C. At 70°C, cyclization activity in pT slowly declined to lower than 20% at 25 minutes while T16 lost most of its activity at 10 minutes (Figures 36, 37). Both enzymes could be stabilized at 70°C in the presence of 2% starch, retaining up to 50% after 60 minutes.

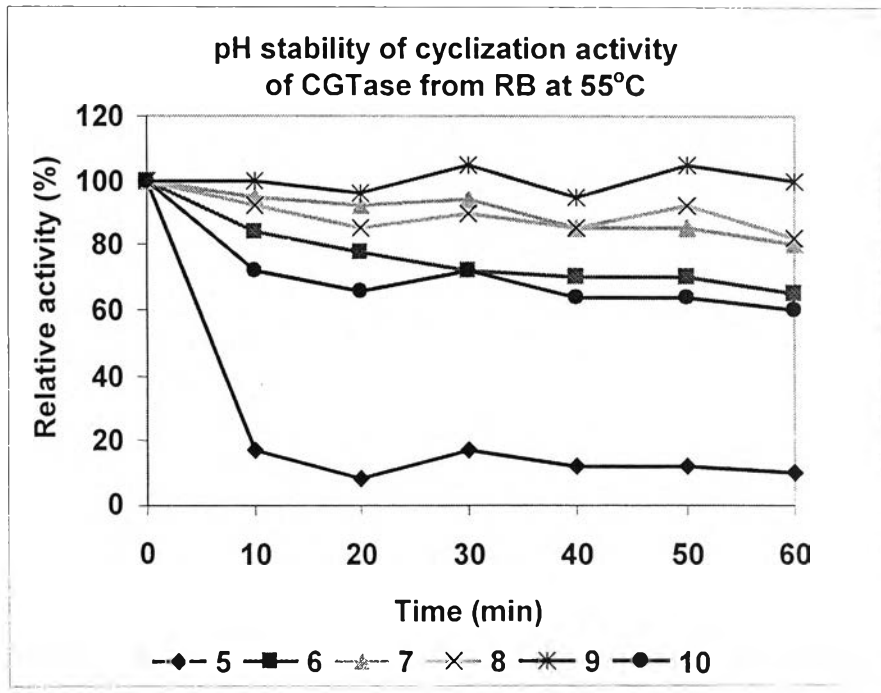


Figure 30 pH stability of cyclization activity of CGTase from RB01.

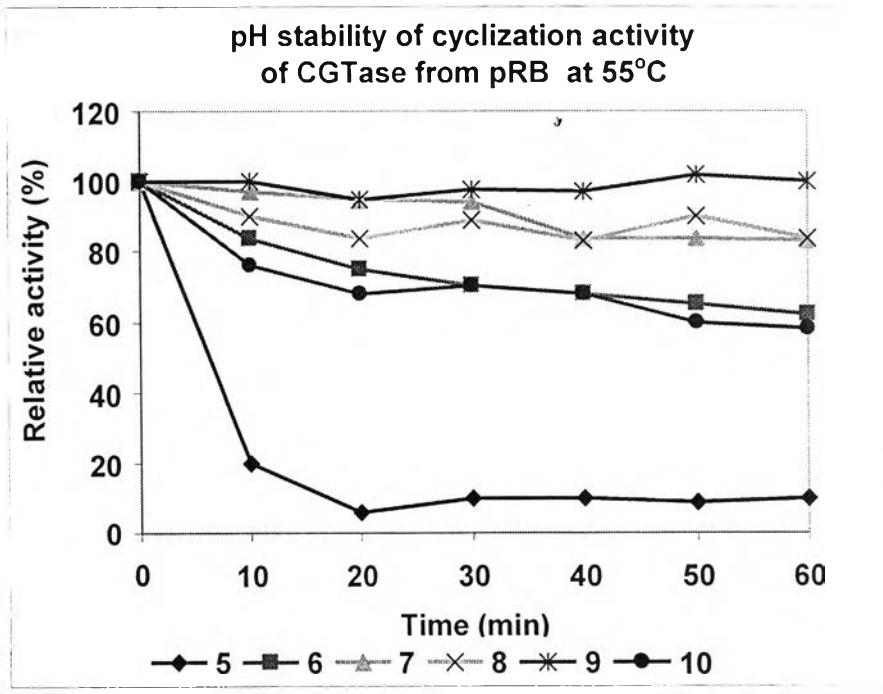


Figure 31 pH stability of cyclization activity of CGTase from pRB.

The purified CGTase was preincubated in various pH's at 55°C for one hour prior to the cyclization assay.

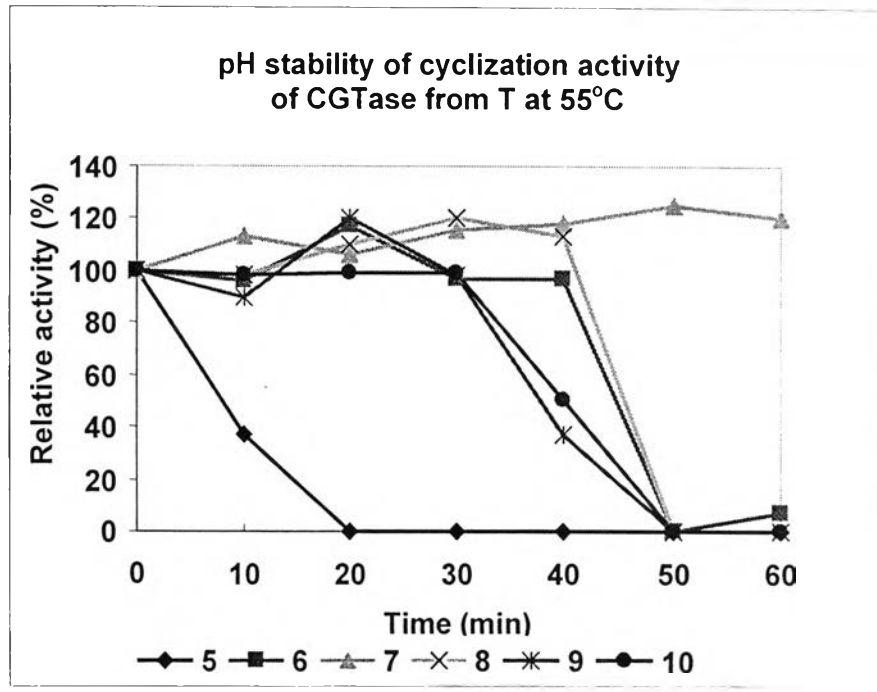


Figure 32 pH stability of cyclization activity of CGTase from T16.

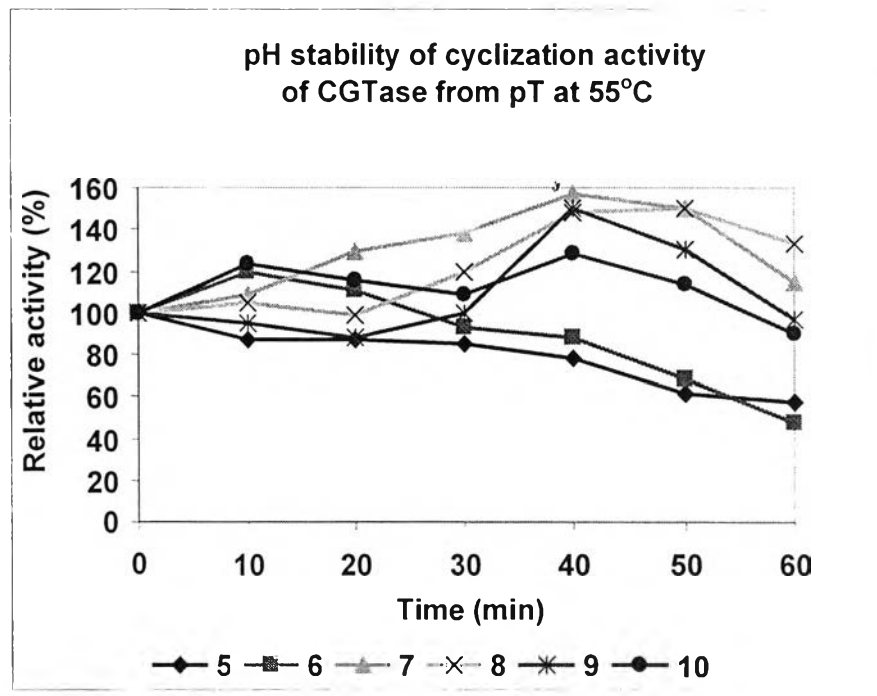


Figure 33 pH stability of cyclization activity of CGTase from pT.

The purified CGTase was preincubated in various pH's at 55°C for one hour prior to the cyclization assay.

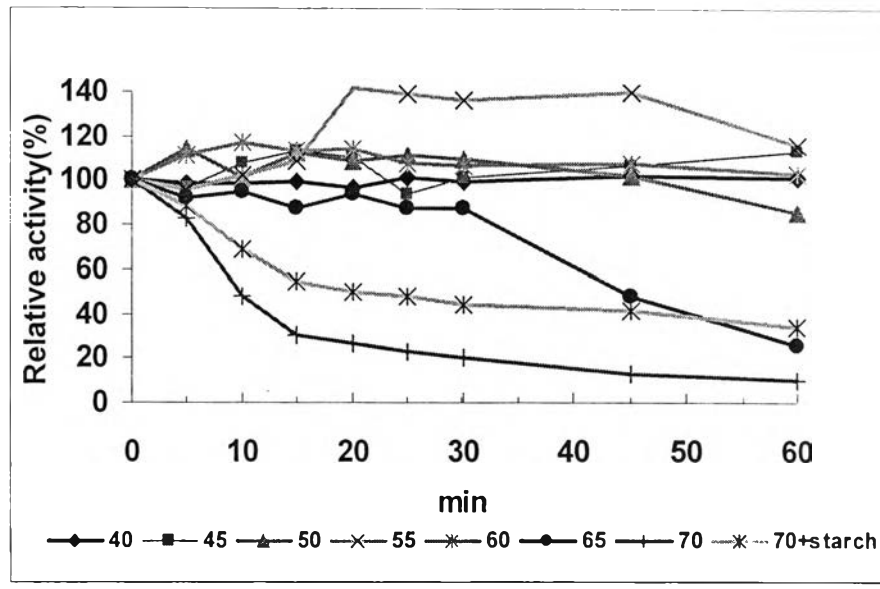


Figure 34 Thermostability of cyclization activity of CGTase from RB at pH 7.0

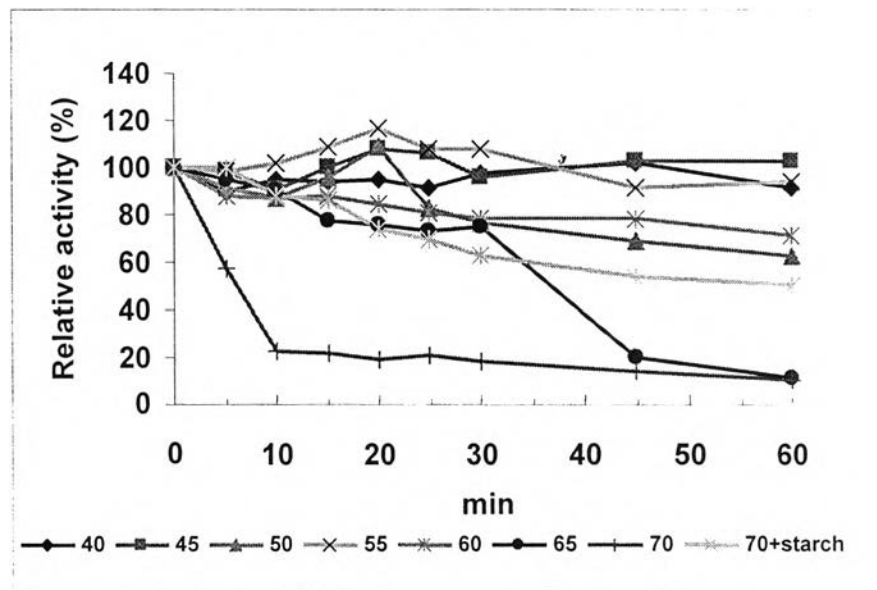


Figure 35 Thermostability of cyclization activity of CGTase from pRB at pH 7.0

The purified CGTase was preincubated in various temperature from 40 to 70°C for one hour prior to the cyclization assay with 200 units of enzyme at 60°C for 30 minutes.

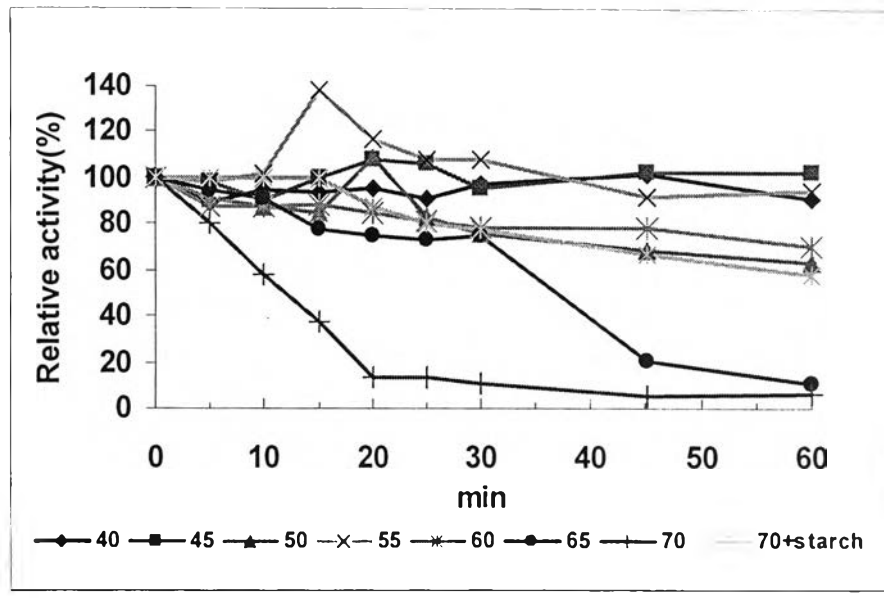


Figure 36 Thermostability of cyclization activity of CGTase from T16 at pH 7.0

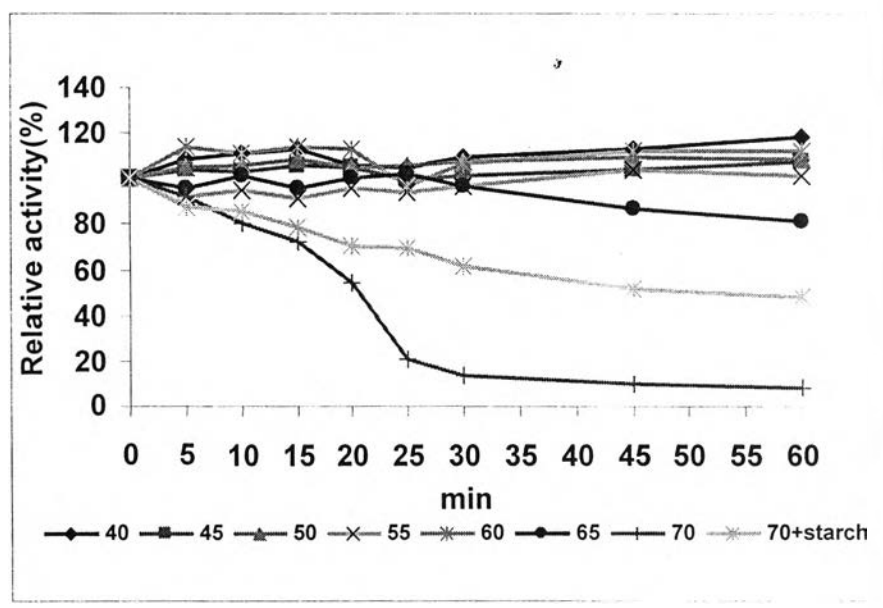
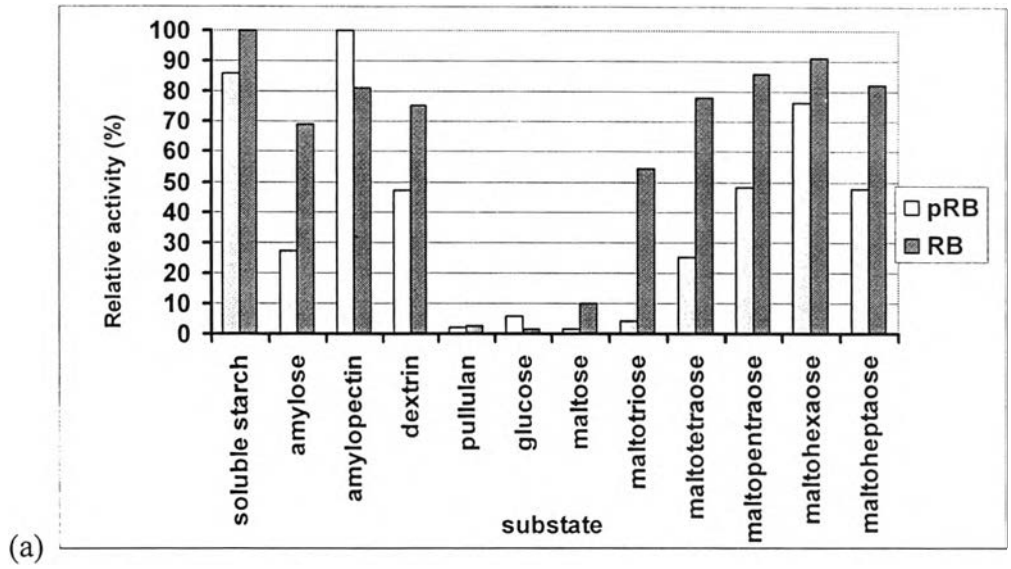


Figure 37 Thermostability of cyclization activity of CGTase from pT at pH 7.0

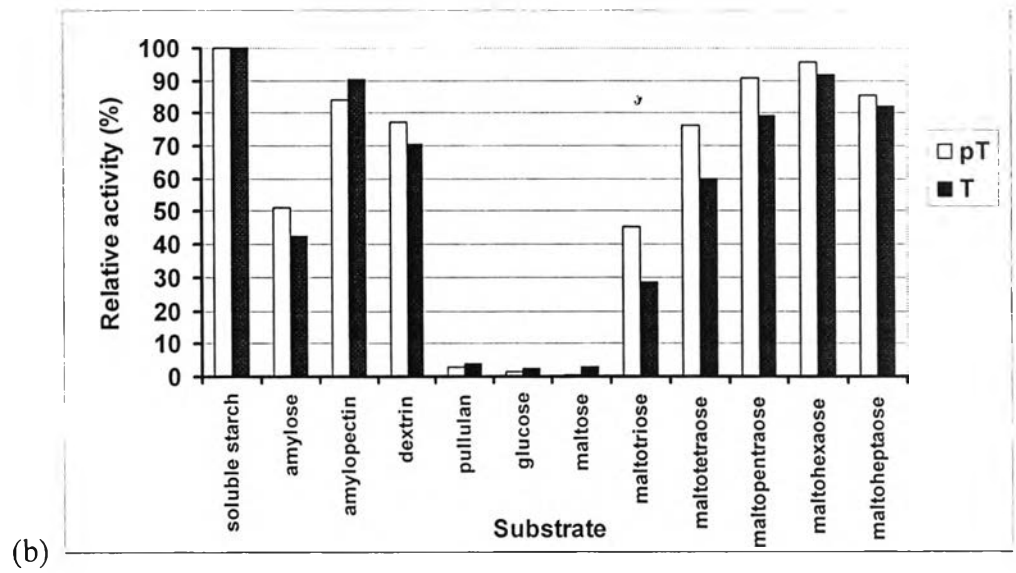
The purified CGTase was preincubated in various temperature from 40 to 70°C for one hour prior to the cyclization assay with 200 units of enzyme at 60°C for 30 minutes.

3.5.5 Substrate specificity of CGTase

Different types of substrate were incubated with CGTase for 30 min at 60°C, and the cyclization activity was measured. Enzymes from RB01, pRB, T16 and pT showed similar substrate specificity (Figure 38). Maximum activity was observed with 6% w/v of soluble starch and 1.5% amylopectin was a better substrate than 6% amylose. Pullulans, glucose and maltose (all at 6%) were the poorest substrates. Only 1.5% w/v concentration of amylopectin was used in this experiment because of its low solubility but it attended 80% relative activity compared to 6% soluble starch. Among the oligosaccharides used (G2-G7), G6 was the best substrate followed by G5 and G7, and G4. Dextrin was as compatible as amylopectin as substrate for CGTase.



(a)



(b)

Figure 38 Substrate specificity of purified CGTases.

(a) RB01 and pRB (b) T16 and pT

Cyclization activity was measured with 200 units of enzyme. Substrates were used at 6.0%W/V (except for amylopectin of which 1.5%) in acetate buffer, pH 6.0 incubated at 60°C for 30 minutes.

Study of some kinetics parameters

Kinetics parameters of the coupling reaction catalyzed by the four CGTase were determined and compared. Based on previous study on the coupling reactions, cellobiose was the only one among the natural occurring sugars tested that acted as a good sugar acceptors. Cellobiose was, therefore, employed as the glycosyl acceptor for the study on kinetic parameters of the coupling reactions with several cyclodextrins as oligosaccharide donors. Experiments were performed in the excess amount of cellobiose (10mM) and varying concentrations of several CDs (α-, β-, γ-CD, glucosyl-α-CD (G-α-CD), glucosyl-β-CD (G-β-CD), and hydroxypropyl-β-CD (HP-β-CD)). The reaction was performed and monitored as described in section 2.15. The results were subjected to Lineweaver-Burk plot and K_m, V_{max}, K_{cat} were determined.

The Lineweaver-Burk Plot and kinetic parameters of coupling reaction of CGTases from RB01 and pRB were shown in Figure 39 and Table 8. The K_m values for the wild type enzyme were in the order HP-β-CD > α-CD > G-α-CD > γ-CD > G-β-CD > β-CD. The k_{cat}/ K_m rate were in the order HP-β-CD > G-β-CD > β-CD > G-α-CD > γ-CD ≅α-CD. The transformant enzyme showed improved K_m for most of the CDs tested except γ-CD which did not change much and much higher K_m for α-CD. K_m values for CDs of the transformant, pRB were in the order α-CD > γ-CD >β-CD> G-α-CD > HP-β-CD > G-β-CD with k_{cat}/ K_m values in the order of HP-β-CD > G-β-CD > β-CD> G-α-CD > γ-CD > α-CD.

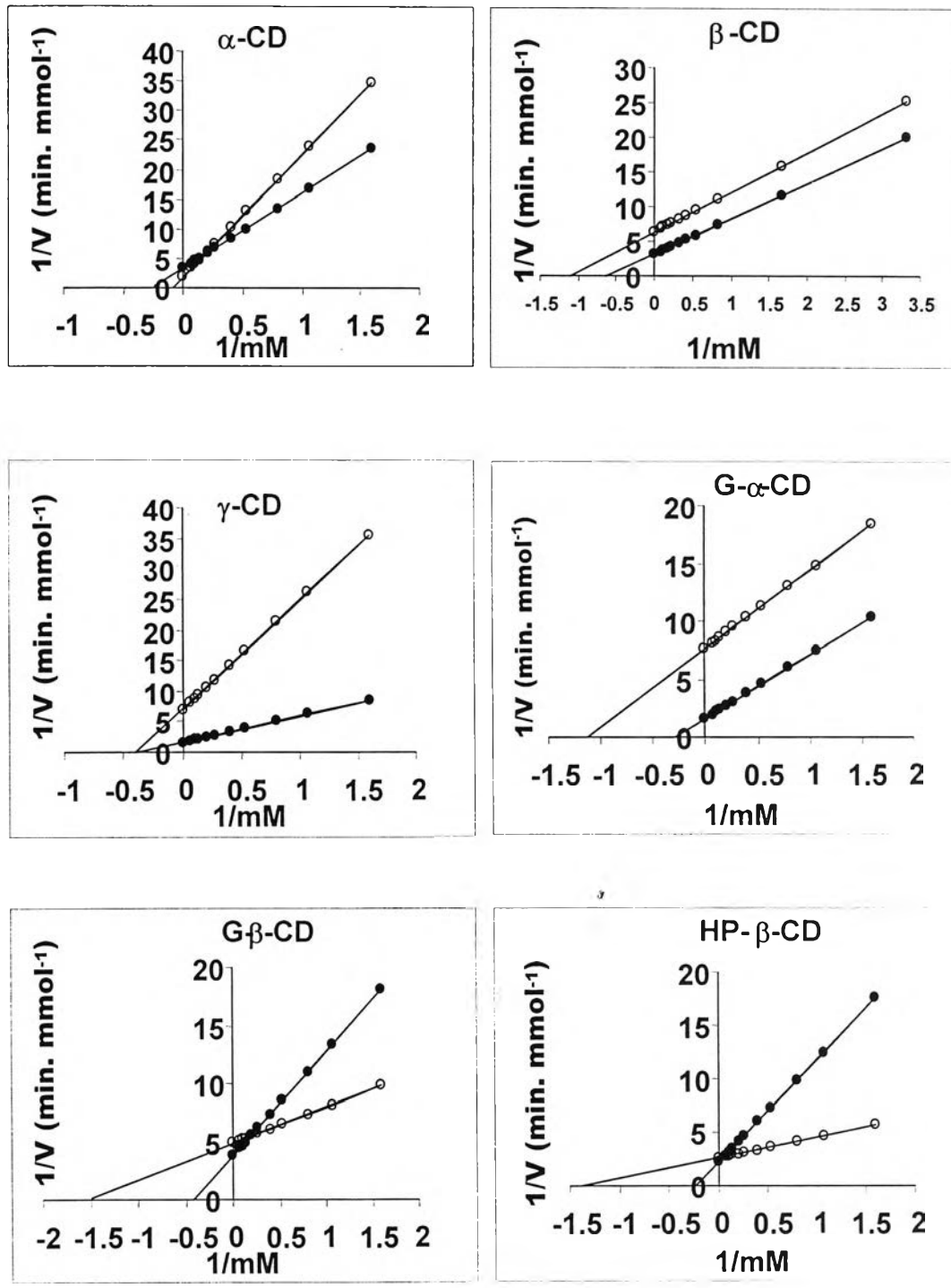


Figure 39 Lineweaver-Burk plot of coupling reaction of CGTases.

RB01 (●) and pRB (○). Cellobiose and various cyclodextrins were used as substrates. CDs (donor) concentrations were varied (0.5-15 mM) for incubation with 10 mM cellobiose (acceptor) for 5 minutes at 55°C.

Table 8 Kinetic parameters of coupling reaction of CGTase from RB01 and pRB.

Cellobiose and various cyclodextrins were used as substrate.

Substrate	K_m (mM)		V_{max} (mM min^{-1} mg^{-1})		k_{cat} (sec^{-1})		k_{cat}/K_m (mM^{-1} sec^{-1})	
	RB01	pRB	RB01	pRB	RB01	pRB	RB01	pRB
α-Cyclodextrin	3.777 ± 0.196	10.443 ± 2.457	0.300	0.509	0.022	0.037	0.006	0.004
β-Cyclodextrin	1.620 ± 0.134	0.887 ± 0.135	0.322	0.156	0.024	0.012	0.015	0.013
γ-Cyclodextrin	2.574 ± 0.660	2.527 ± 0.160	0.608	0.142	0.004	0.011	0.002	0.004
Glucosyl-α-Cyclodextrin	3.423 ± 0.401	0.876 ± 0.220	0.624	0.131	0.046	0.009	0.013	0.011
Glucosyl-β-Cyclodextrin	2.385 ± 0.231	0.634 ± 0.050	0.265	0.206	0.002	0.015	0.001	0.024
Hydroxypropyl-β-Cyclodextrin	4.445 ± 0.298	0.718 ± 0.044	0.462	0.383	0.034	0.028	0.008	0.039

* k_{cat} were calculated from the formula, V_{max} Divided by the Total Enzyme Concentration .

Figure 40 and Table 9 showed the results for enzymes from T16 and pT16. T16 showed highest affinity (lower K_m) for HP-β-CD followed by G-α-CD, γ-CD, β-CD , G-β-CD and α-CD respectively. CGTase from pT16 also showed highest affinity for HP-β-CD while the order of affinity slightly changed. The enzyme from pT seemed to have improved affinity for β-CD and its glucosyl derivative. The k_{cat}/ K_m ratios of pT enzyme also seemed to be much improved for β-CD and also for HP-β-CD.

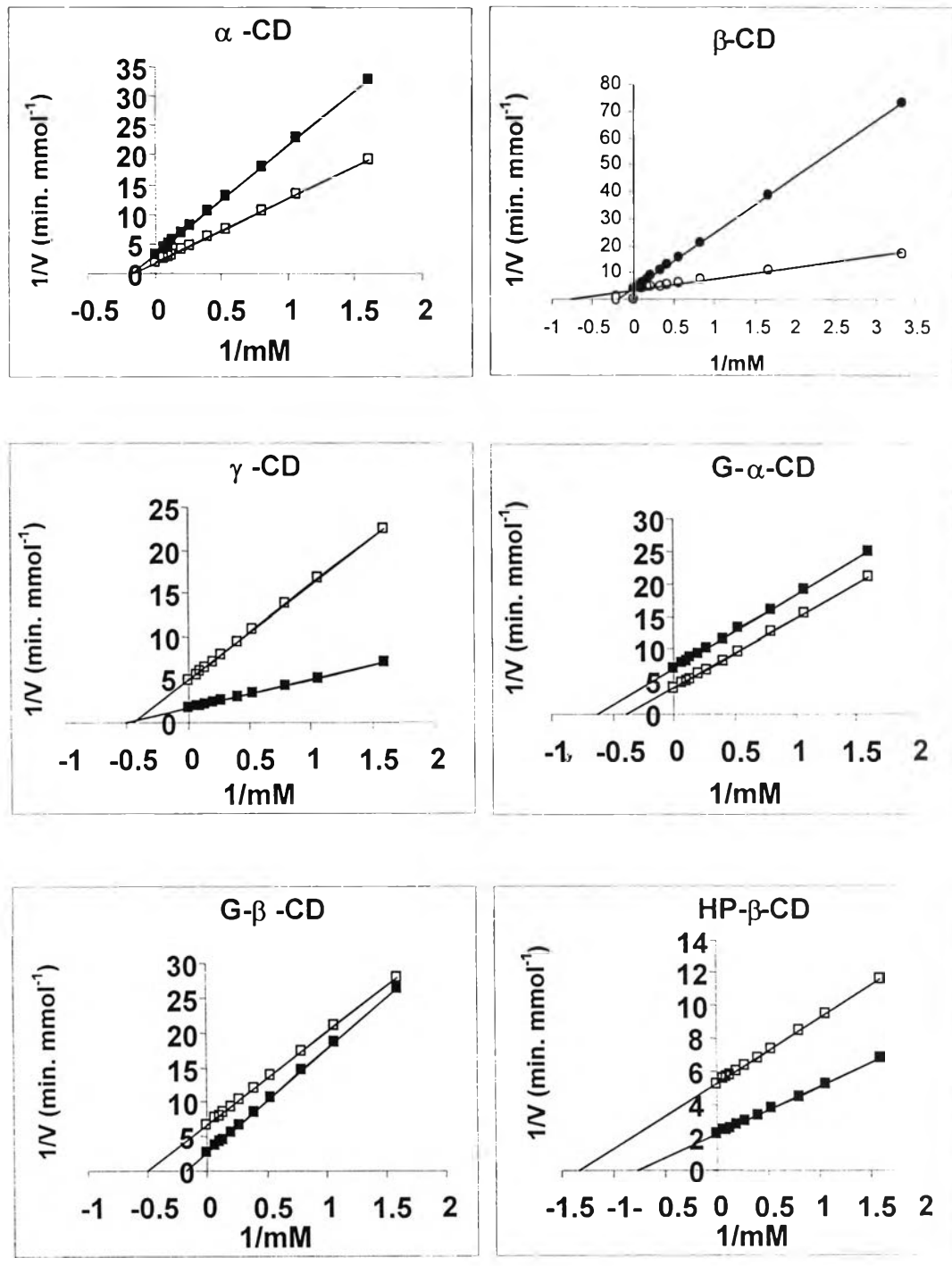


Figure 40 Lineweaver-Burk plot of coupling reaction of CGTases.

T16 (■) and pT (□). Cellobiose and various cyclodextrins were used as substrates. CDs (donor) concentrations were varied (0.5-15 mM) for incubation with 10 mM cellobiose (acceptor) for 5 minutes at 55°C.

Table 9 Kinetic parameters of coupling reaction of CGTase from T16 and pT.

Cellobiose and various cyclodextrins were used as substrate.

Substrate	K_m (mM)		V_{max} (mM min^{-1} mg^{-1})		k_{cat} (sec^{-1})		k_{cat}/K_m (mM^{-1} sec^{-1})	
	T16	pT	T16	pT	T16	pT	T16	pT
α-Cyclodextrin	6.048 ± 0.908	6.299 ± 1.956	0.325	0.577	0.003	0.044	0.001	0.007
β-Cyclodextrin	5.266 ± 0.975	1.099 ± 0.506	0.254	0.256	0.002	0.020	0.001	0.018
γ-Cyclodextrin	2.082 ± 0.298	2.261 ± 0.395	0.623	0.205	0.093	0.016	0.044	0.007
Glucosyl-α-Cyclodextrin	1.595 ± 0.236	2.615 ± 0.982	0.142	0.248	0.021	0.019	0.013	0.007
Glucosyl-β-Cyclodextrin	5.636 ± 0.562	1.963 ± 0.680	0.379	0.148	0.057	0.011	0.010	0.006
Hydroxypropyl-β-Cyclodextrin	1.291 ± 0.318	0.752 ± 0.582	0.453	0.191	0.004	0.015	0.003	0.019

* k_{cat} were calculated from the formula, V_{max} Divided by the Total Enzyme Concentration.

3.5.7 Product analysis by High Performance Liquid Chromatography

The reaction mixture containing 2.5 ml of 2.0% soluble starch in 0.2 M phosphate buffer, pH 6.0 was incubated with 0.5 ml enzyme at 40°C for 24 h and reaction was stopped by incubation in boiling water bath for 10 min. β-amylase (20 U/ml) was added to the aliquot and the mixture was incubated at 25°C for 3 hours. The HPLC analysis of the resulted mixture revealed the presence of mainly 2 types of CDs. It was found that CGTase from wild types and transformants produced mainly β-CD. However, T16 gave almost equal amount of all three CDs with slightly higher amount of γ-CD. The ratio of α: β: γ-CD was 0.57 : 1 : 0.13 for RB01, 0.21 : 1 : 0.05 for pRB, 0.83 : 1 : 1.1 for T16 and 0.25 : 1 : 0.51 for pT (Figure 40 and Table 10)

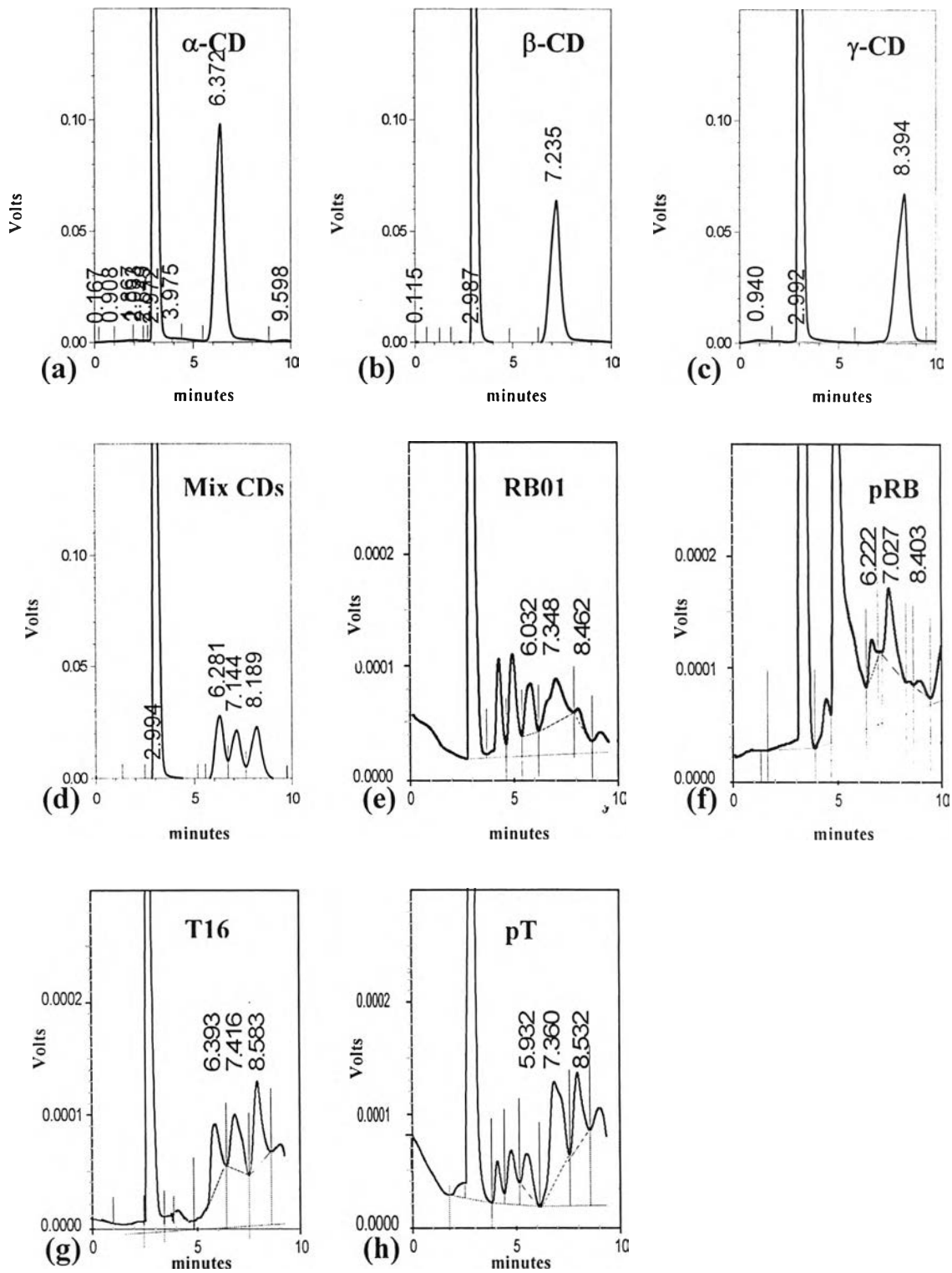


Figure 41 HPLC chromatogram of CDs produced by purified CGTase and standard cyclodextrins (α-CD, β-CD and γ-CD).

(a-d) standard CD, e-h) CGTase from RB, pRB, T16 and pT, respectively. Lichrocart-NH_2 column was used. Acetonitrile : water (70 : 30, v/v) was used as eluent at 1.6 ml/min flow rate.

Table 10 CD products of purified CGTase determined by HPLC.

	Area Peak			Ratio
	α-CD	β-CD	γ-CD	α : β : γ
RB01	5.88	10.28	1.38	0.57 : 1 : 0.13
pRB	5.65	27.44	1.35	0.21 : 1 : 0.05
T16	3.294	3.45	5.41	0.95 : 1 : 1.57
pT	2.96	11.66	5.95	0.25 : 1 : 0.51