CHAPTER III

RESULTS

Purification of CGTase

Crude CGTase was purified by three major consecutive steps, namely, corn starch adsorption, precipitation at 40-80% ammonium sulfate saturation, and DEAE-cellulose column chromatography. The first two steps repeat those reported by Rojtinnakorn (1994) and the results obtained were similar. DEAE-cellulose column chromatography was modified by narrow down the range of NaCl gradient used. Rojtinnakorn (1994) used 0-1 M NaCl (200+200 ml), a rather steep gradient of NaCl as eluant for CGTase. In this work, 0-0.3 M NaCl (200+200 ml) was used.

The column profile (Figure 7) shows only one single protein peak, eluted between fraction 60 to 120 which coincided with dextrinizing activity peak. The highest dextrinizing activity was obtained at the same position as the protein peak which was eluted at the salt concentration of approximately 0.1 M NaCl. The fractions were pooled as separate pools, as followed: fraction number 60-67, 69-71, 73-75, 76-79, 80, 82-84, 86-92, 93-98, 99-104, and 105-120, which were named pool E1-E10, respectively.

Pool E1-E10 were analyzed by non-denaturing PAGE. When stained with protein staining (Figure 8C), E1-E4 showed 3-4 faint protein bands. E5-E8 showed 2 bands (band a, b)with the faster being less in intensity

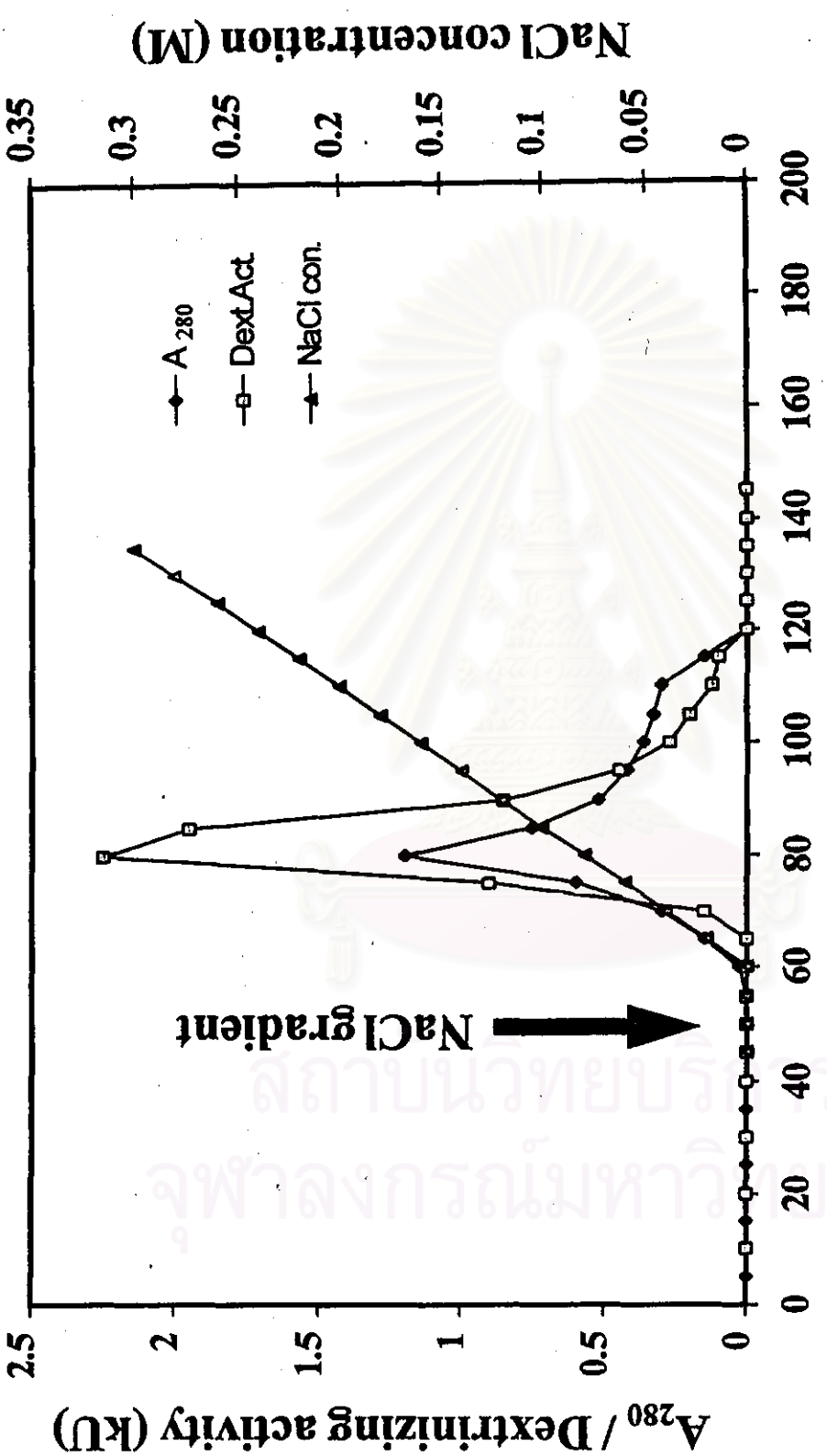


Figure 7 Profile of DEAE-cellulose column chromatography of CGTase, from *Bacillus* sp.A11 at pH 8.5. Column size 2.2x22 cm, flow rate 30 ml/hr, and fraction size 3 ml were conditions used.

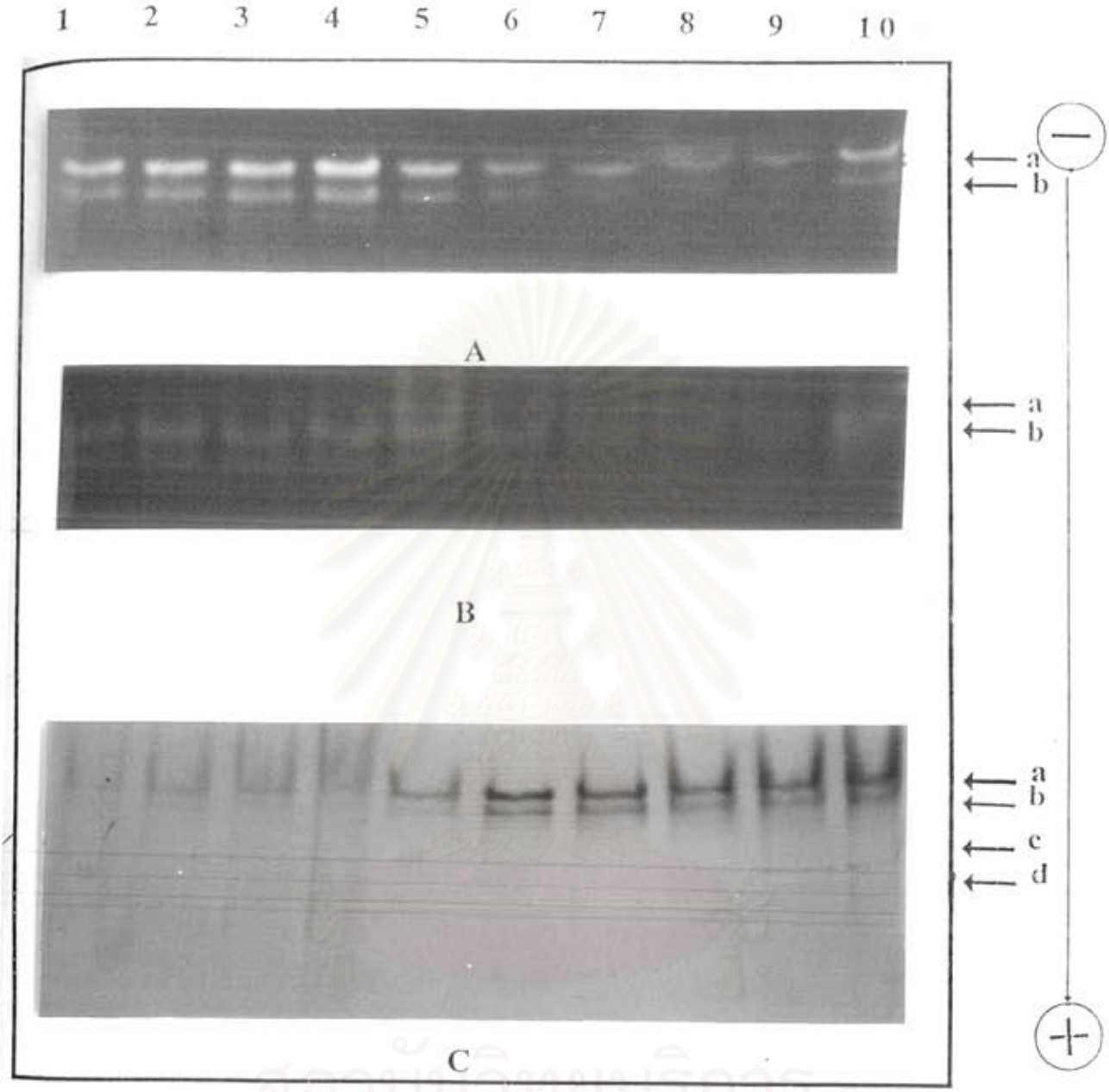


Figure 8 Non-denaturing PAGE. of fractions obtained from DEAE-cellulose column chromatography

Lane 1 to 10 were pools E1 to E10, respectively.
A. Amylolytic activity staining by iodine solution
(0.2 units of Dextrinizing activity was subjected to each well)
B. CD-forming activity staining
(2.0 units of Dextrinizing activity was subjected to each well)
C. Coomassie blue protein staining
(2 μg protein was subjected to each well)

whereas pool E9-E10 showed 3-4 protein bands (band a, b, c, d). Activity stain (both amylolytic and CD-forming) of all pools in Figure 8A and 8B showed two major bands (a and b) of different intensity corresponded to bands from protein staining. SDS-PAGE of pool E5-E7 showed one major protein band while pool E4, E8-E10 showed other faint bands beside the same major band. E1-E3 had several faint bands with no major band observed (Figure 9). Pool E5-E7 were then pooled and called DEAE-cellulose enzyme.

The recovery and yield of CGTase obtained at each purification step expressed in terms of dextrinizing activity are shown in Table 11. Specific activity of dextrinization was increased through each step. These corresponded to CD-product which was determined by CD-TCE dilution limit value. After the final step of the purification, yield of 30% was obtained with 44.8 fold of purity.

Purification of CGTase was demonstrated by non-denaturing and SDS-polyacrylamide gel electrophoresis. In the normal PAGE (Figure 10A), although the protein bands were faintly detected in the crude enzyme of *Bacillus* sp. A11, the subsequent purification up to DEAE-cellulose chromatography efficiently separated the CGTase candidates and concentrated for the final purification. The DEAE-cellulose could further separate proteins band a and b from the others. These two bands clearly exhibited the amylolytic activity when stained with iodine solution as shown in Figure 10B. When the similar gel was stained for CD products (Figure 10C), CD were detected at the positions of proteins band a and b in each step of purification.

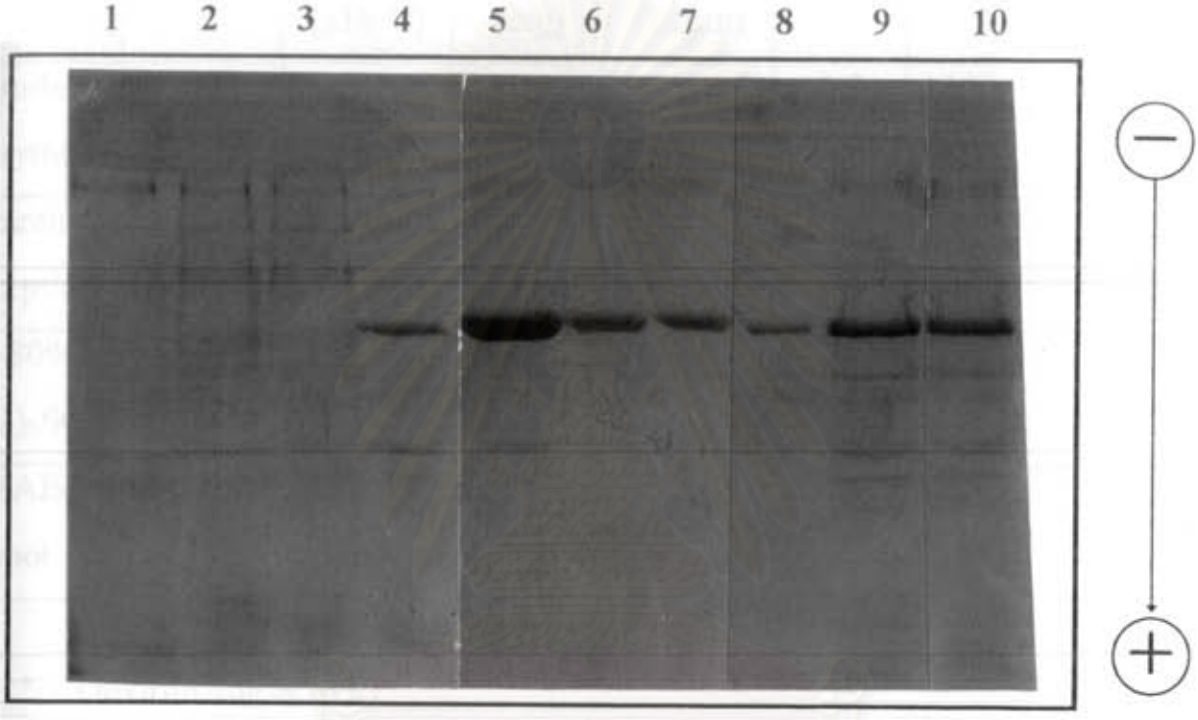


Figure 9 SDS-PAGE of fractions obtained from DEAE-cellulose column chromatography (2.0 μg protein was subjected to each well, Lane 1 to 10 were pools E1 to E10, respectively.) Protein staining was performed using coomassie blue.

Table 11 Purification table of CGTase from *Bacillus* sp. A11

Step	Volume (ml)	Total activity* ($x10^3$U)	Total protein (mg)	Specific activity* (U/mg)	Purity (fold)	Yield (%)	Activity (CD-TCE) (2^n)
Crude enzyme	5,000	200	1,790	111.7	1.0	100	2^6
Starch adsorption	1,000	190	410	463.4	4.1	95	2^9
40-80% $(NH_4)_2SO_4$	80	80	50	1,600.0	14.3	40	2^{13}
DEAE-pool	36	60	12	5,000.0	44.8	30	2^{14}

Note * = Dextrinizing activity

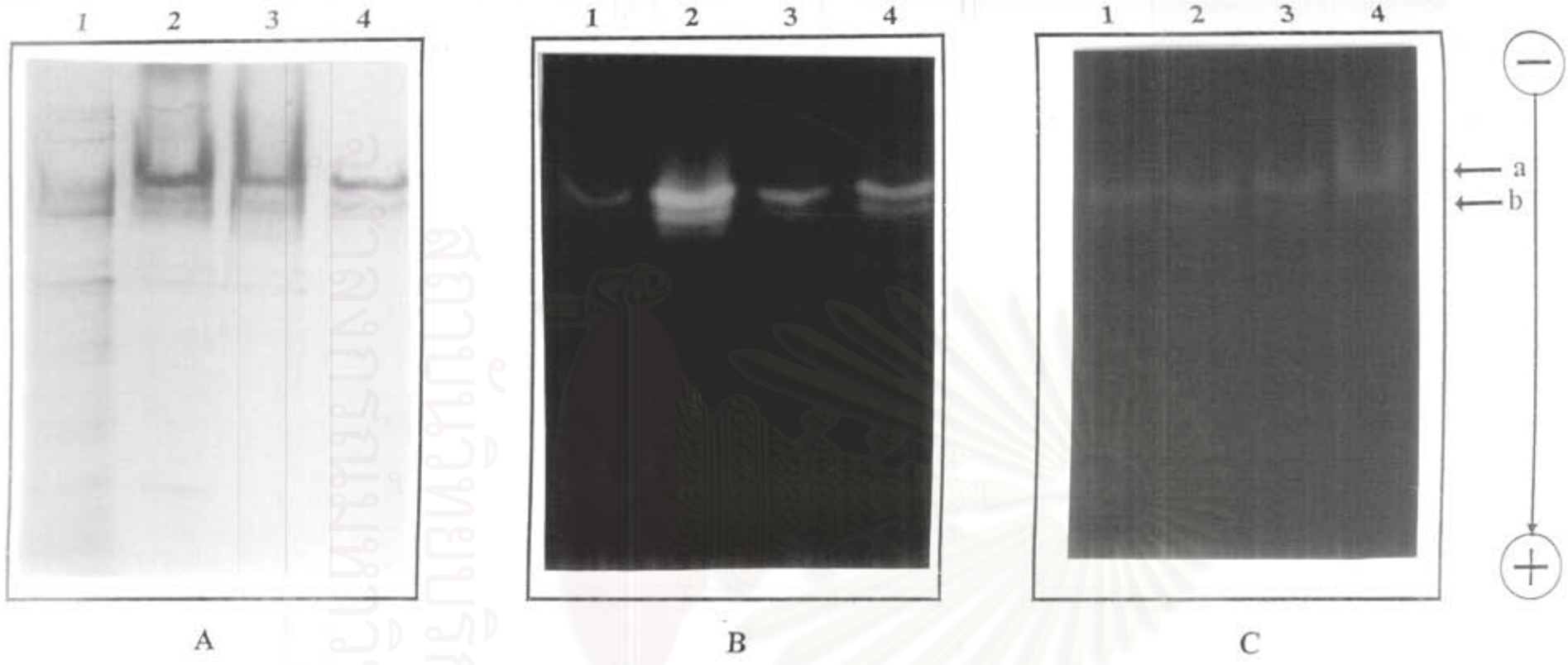


Figure 10 Non- denaturing PAGE of CGTase from different steps of purification

A: Coomassie blue staining [Lane 1. Crude enzyme (20 μg), 2. Starch adsorbed enzyme (20 μg), 3. $(NH_4)_2SO_4$ fraction (5 μg), and 4. DEAE-cellulose pool (2 μg)], B: Amylolytic activity staining by iodine solution [Lane 1-4, as in A, 0.2 units of Dextrinizing activity was subjected to each well], and C: CD-forming activity staining [Lane 1-4, as in A, 2 units of Dextrinizing activity was subjected to each well)]

In the SDS-PAGE (Figure 11), only one intense band was observed in the DEAE-cellulose pool. This protein, assumingly CGTase, was further estimated for its molecular weight. Figure 12 shows the R_f values of standard proteins calculated from distance measured in the SDS-PAGE plotted against the log molecular weight values. The molecular weight of CGTase was estimated to be approximately 72,000 daltons.

Amino acid composition of CGTase

The DEAE-cellulose enzyme was hydrolyzed with either 6M HCl or 4M methanesulfonic acid (for tryptophan detection) at 110 ^{0}C for 22 hours. For analysis of cysteine and methionine, the enzyme was hydrolyzed with performic acid for 30 minutes before the addition of 6M HCl. Chromatograms of various amino acid standards and enzyme sample were shown in Figure 13 and 14. The amino acid composition of the purified enzyme of *Bacillus* sp. A11 obtained from DEAE-cellulose column chromatography was presented in Table 12. The result shows that CGTase was rich in Asx, Gly, Ala, Glx, and Thr while contained few amounts of Met, Cys, and Trp, respectively.

N-terminal amino acid sequence of CGTase

200 pmol of the purified enzyme was separated by SDS-PAGE. The gel was removed from the electrophoresis cell and soaked in electroblotting buffer for 5 minutes. The electroblotting was performed with the same buffer under a constant voltage current of 50 volts at room

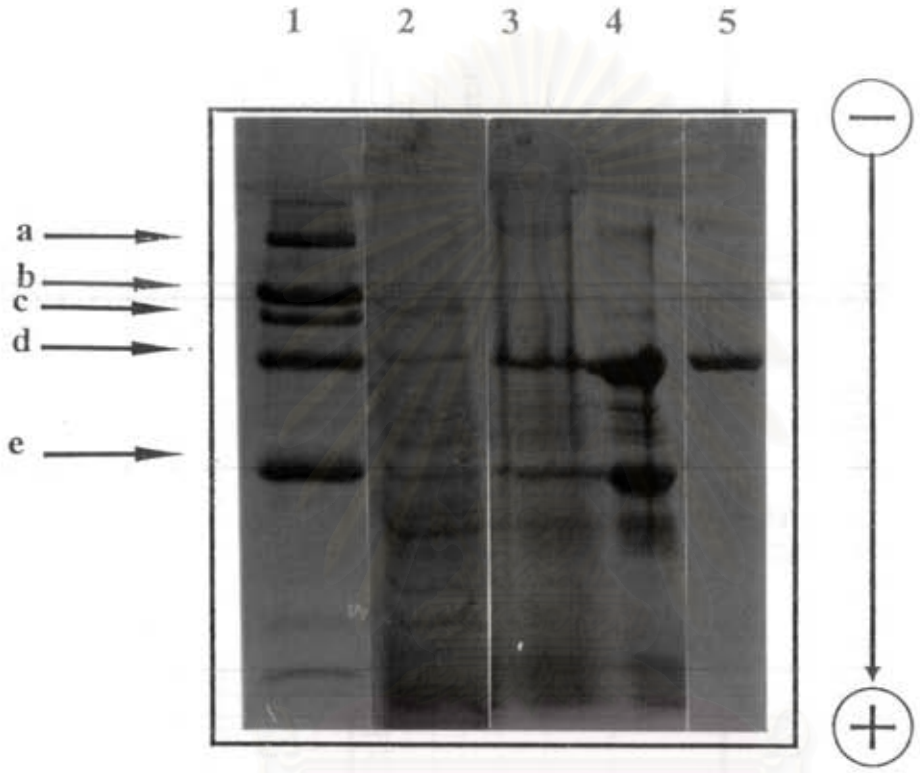


Figure 11 SDS-PAGE of CGTase from different steps of purification

Lane 1. Protein molecular weight markers (a: myosin (200 kD), b: β-galactosidase (116.25 kD), c: phosphorylase (97.1 kD) d: BSA (66.2 kD), and e: ovalbumin (45 kD)

2. Crude enzyme (20 μg)

3. Starch adsorbed enzyme (20 μg)

4. $(NH_4)_2SO_4$ fraction (5 μg)

5. DEAE-cellulose pool (2 μg)

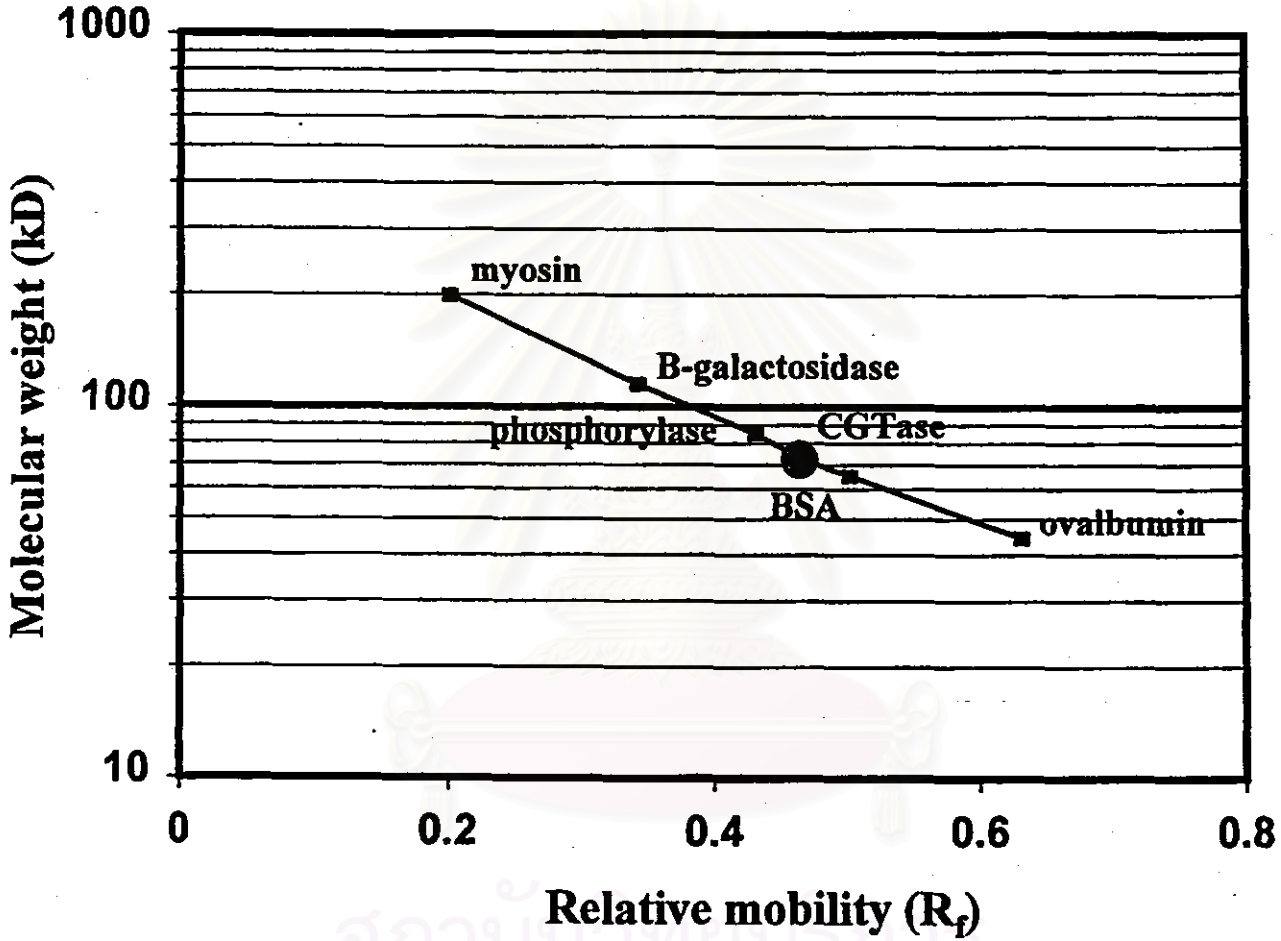


Figure 12 Standard curve of molecular weight and relative mobility from SDS- PAGE technique

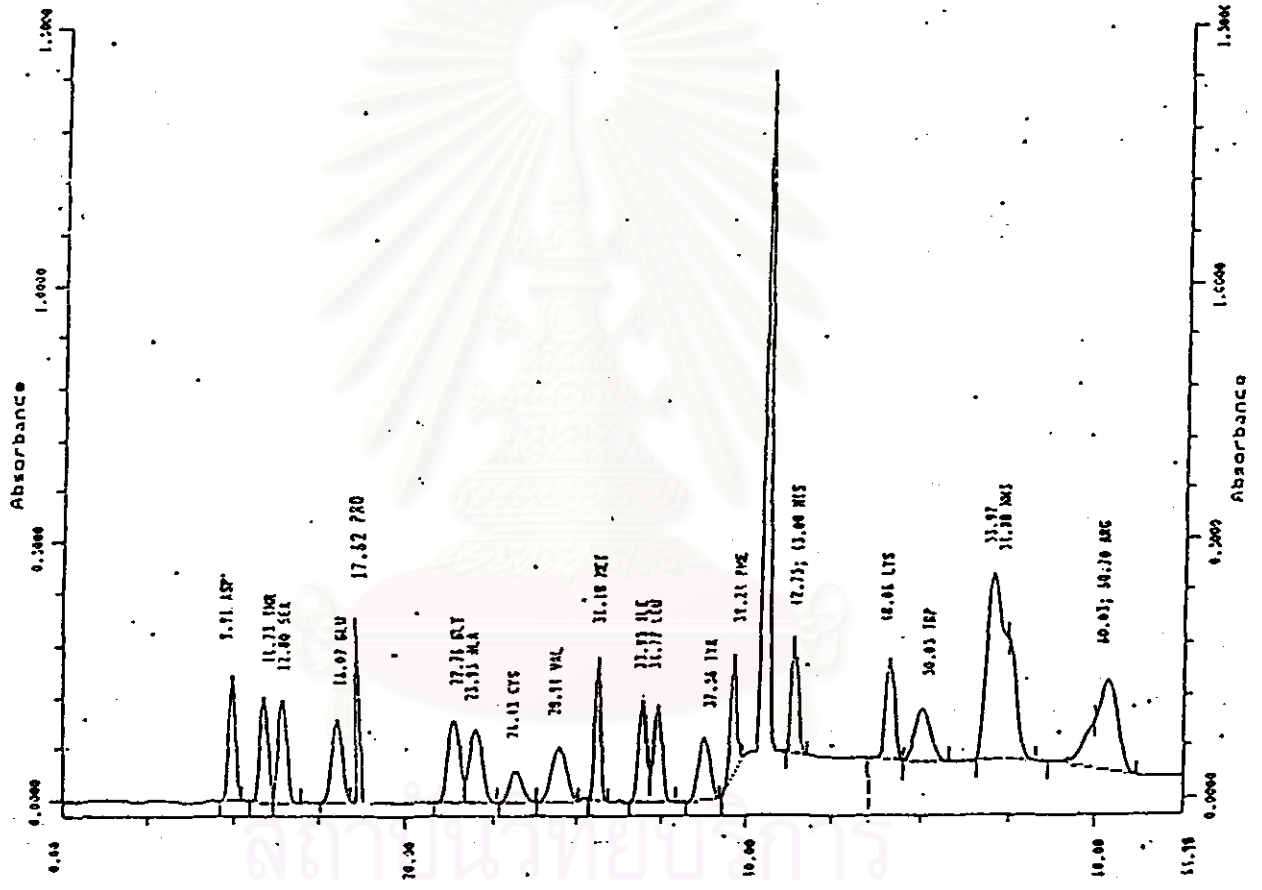


Figure 13 Chromatogram of various amino acid standards from Amino acid analyzer

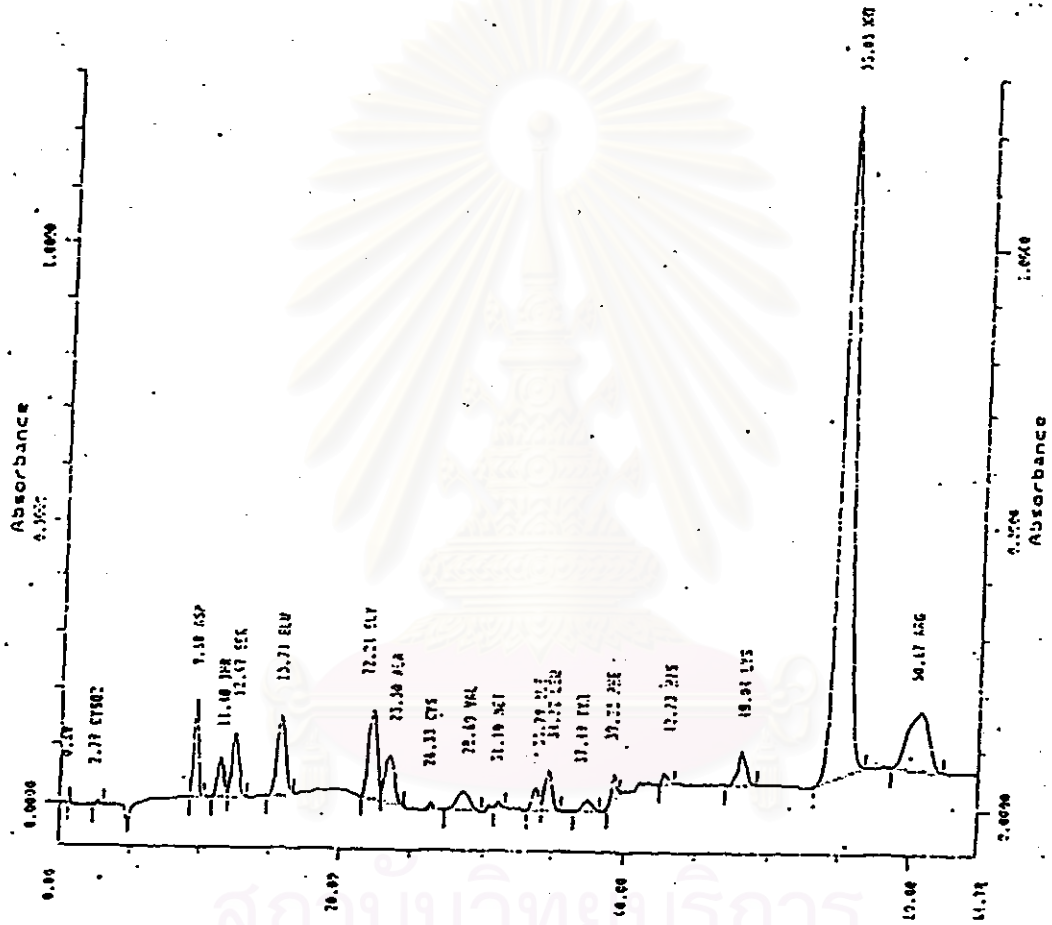


Figure 14 Chromatograms of amino acid composition of CGTase from Amino acid Analyzer

Table 12 Amino acid composition of CGTase from *Bacillus* sp. A11

Amino acid	Content (mol %)
Asx	15.42
Glx	8.63
Ser	6.25
Gly	12.35
His	3.17
Thr	8.31
Arg	2.82
Ala	10.24
Pro	6.46
Tyr	1.55
Val	6.95
Met	0.44
Cys	0.29
Ile	4.28
Leu	5.54
Phe	3.56
Trp	0.16
Lys	3.56
Total	100

Note Asx = aspartic acid + asparagine

Glx = glutamic acid + glutamine

temperature for 30 minutes. After electroblotting, the protein bands in the PVDF membrane was briefly stained by Coomassie blue and destaining with 50% methanol until protein bands appeared (Figure 15). The band of CGTase was excised and subjected for sequencing.

The sequencing procedure for determination of the amino acid residues was as mentioned in Methods section 2.12.3. The twenty cycles were analyzed on an Applied Biosystem PTH-C18 reversed phase cartridge column. Twenty amino acids, aspartic acid (D), asparagine (N), serine (S), glutamine (Q), threonine (T), glycine (G), glutamic acid (E), histidine (H), alanine (A), arginine (R), tyrosine (Y), proline (P), methionine (M), valine (V), tryptophan (W), phenylalanine (F), isoleusine (I), lysine (K), leusine (L), and cysteine (Cys) were used as sequence standards and dmptu (N,N-dimethyl-N′-phenylthiourea) and dptu (diphenylthiourea) peaks were used as reference peaks. The profiles of sequence standards and profiles of amino acid sequence of CGTase were shown in Figure 16 a-l.

The N-terminal amino acid sequence of the purified CGTase was determined by automated Edman degradation up to 20 residue to be A P D T S N Y N K Q N F R T D V I Y Q I.

Oligonucleotide design and synthesis

20 residues of N-terminal amino acid sequence of CGTase from *Bacillus* sp. A 11 was compared with N-terminal amino acid sequence of CGTase from other microorganisms, as shown in Figure 17 and 18. Homologies of 80% amino acid identity was demonstrated. The C-terminal

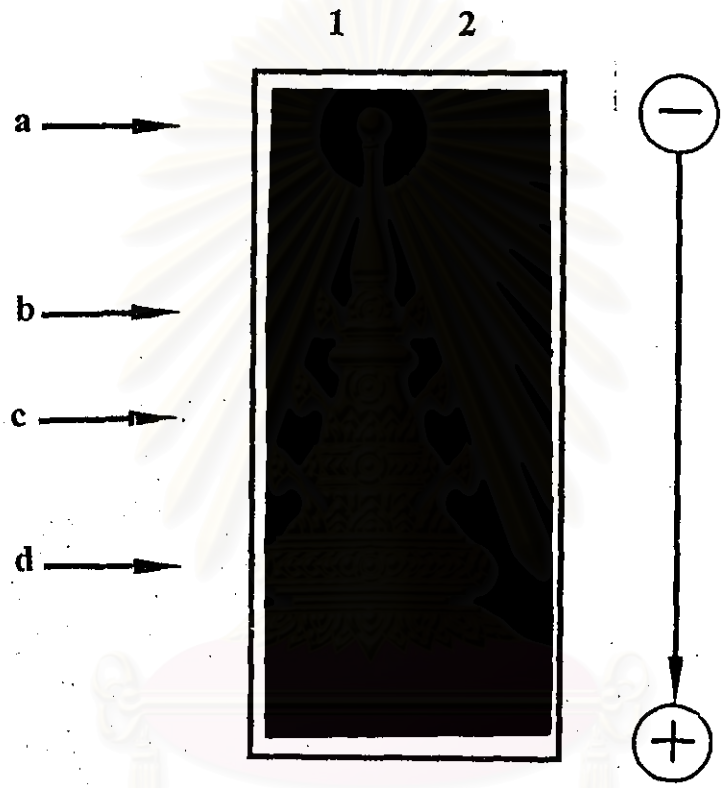


Figure 15 Analysis of CGTase from *Bacillus sp.* A11 after electroblotted onto PVDF membrane

Lane 1: Standard protein markers; a: Myosin (200 kD), b: BSA (66.2 kD), c: Ovalbumin (45 kD), and d: Bovine carbonic anhydrase (29 kD).

Lane 2: Purified CGTase

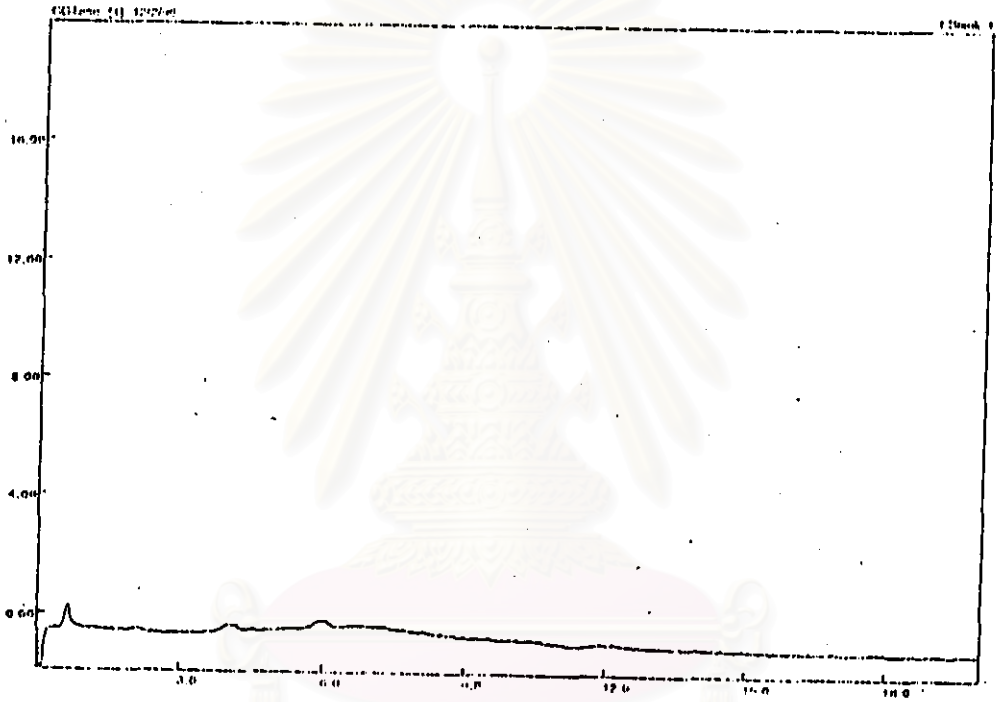


Figure 16a Chromatogram of blank condition obtained from PTH-C18 reverse phase cartridge column

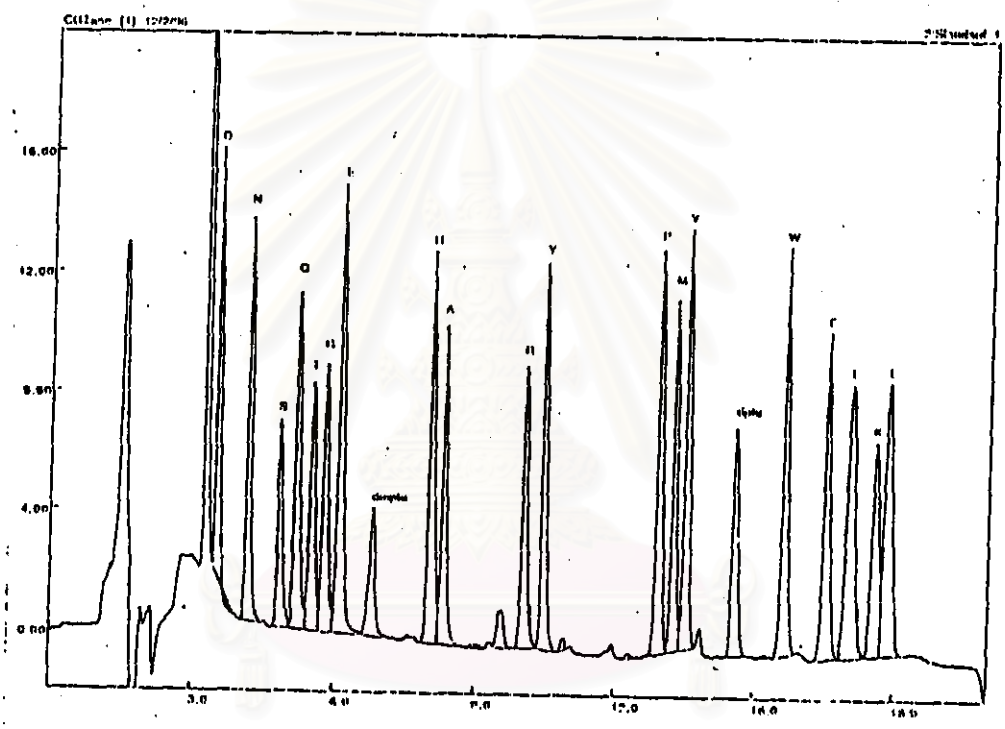
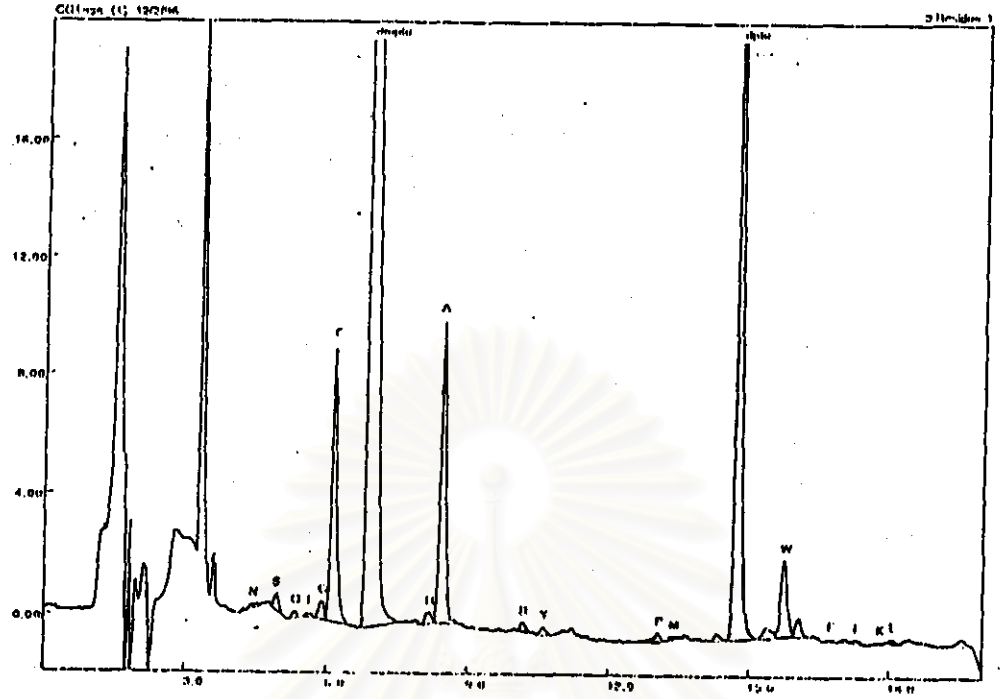
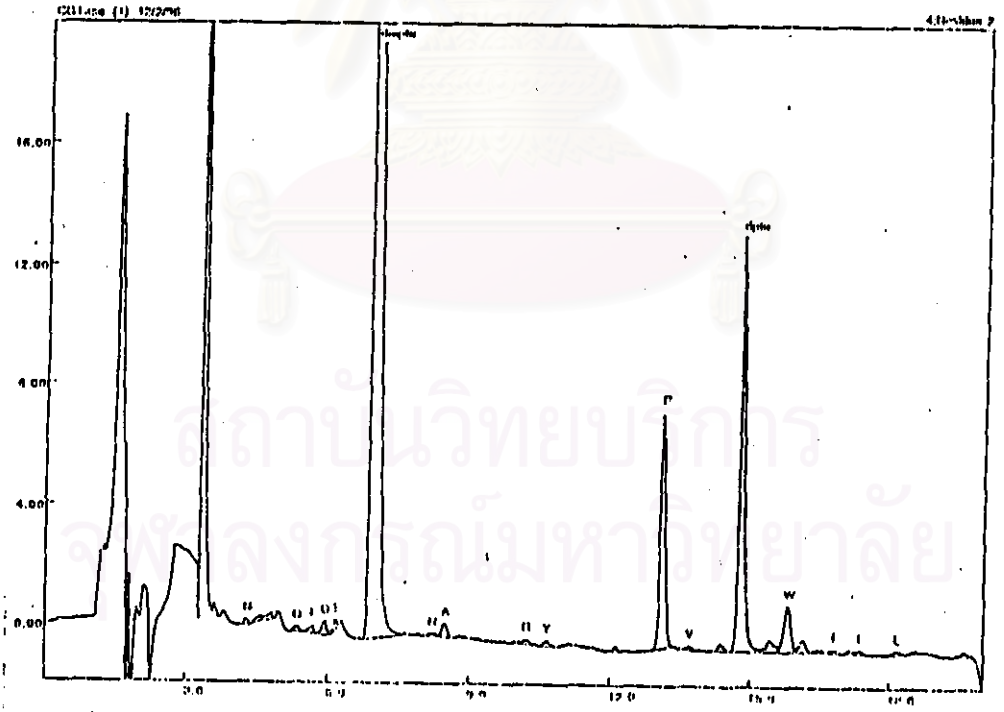


Figure 16b Chromatogram of amino acid standards from PTH-C18 reverse phase cartridge column

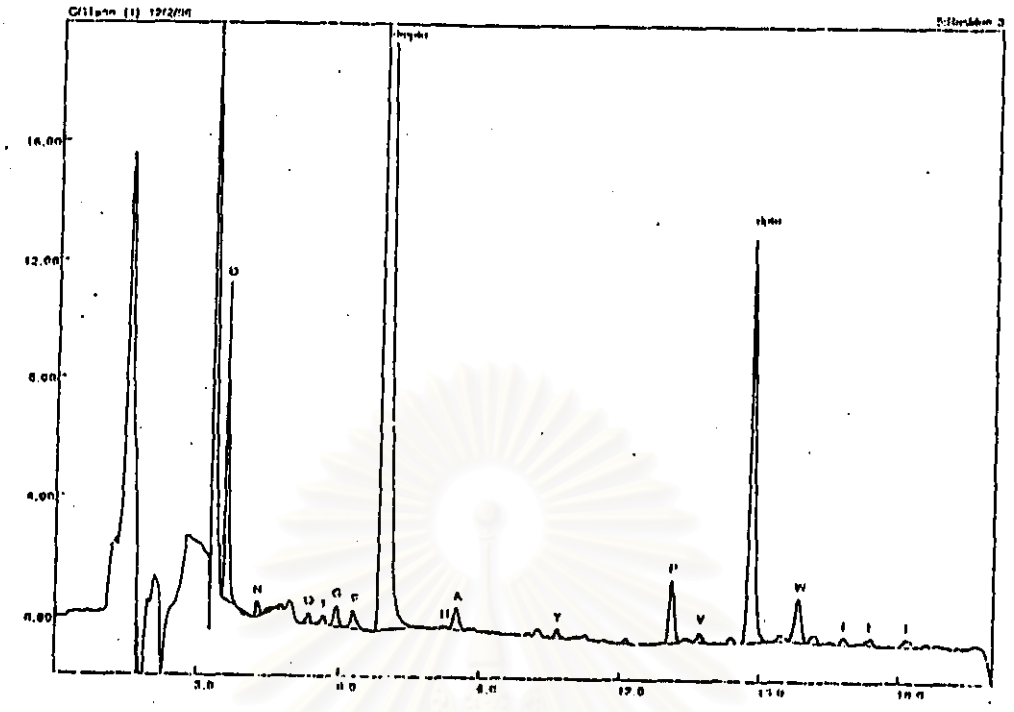


(A)

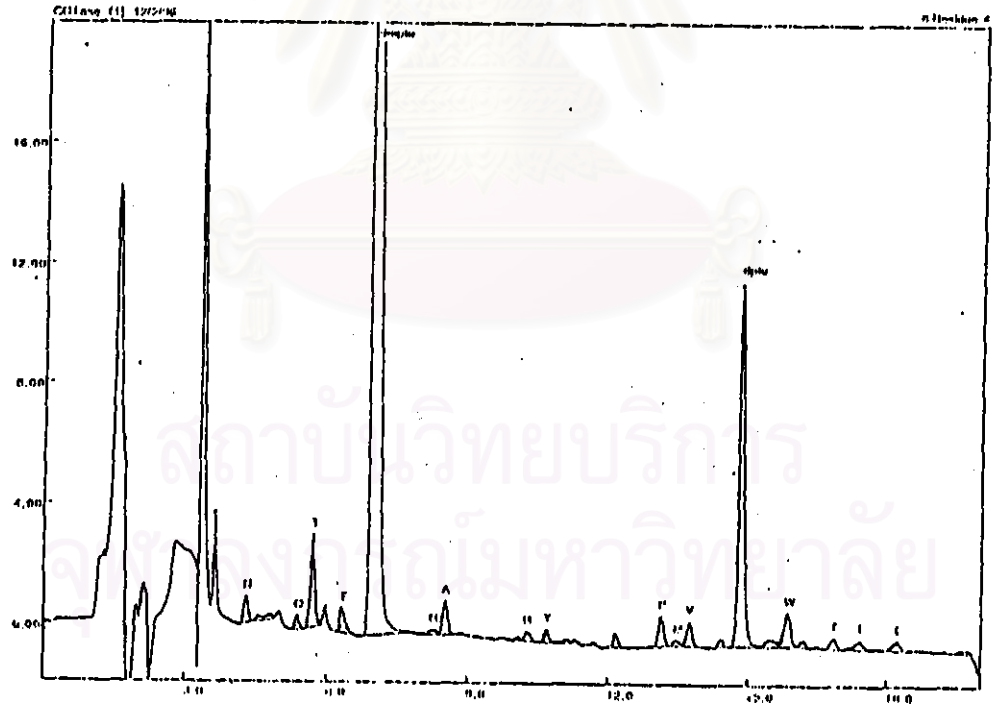


(B)

Figure 16c Chromatogram of N-terminal amino acid of purified CGTase from PTH-C18 reverse phase cartridge column
(A) Cycle 1 (B) Cycle 2

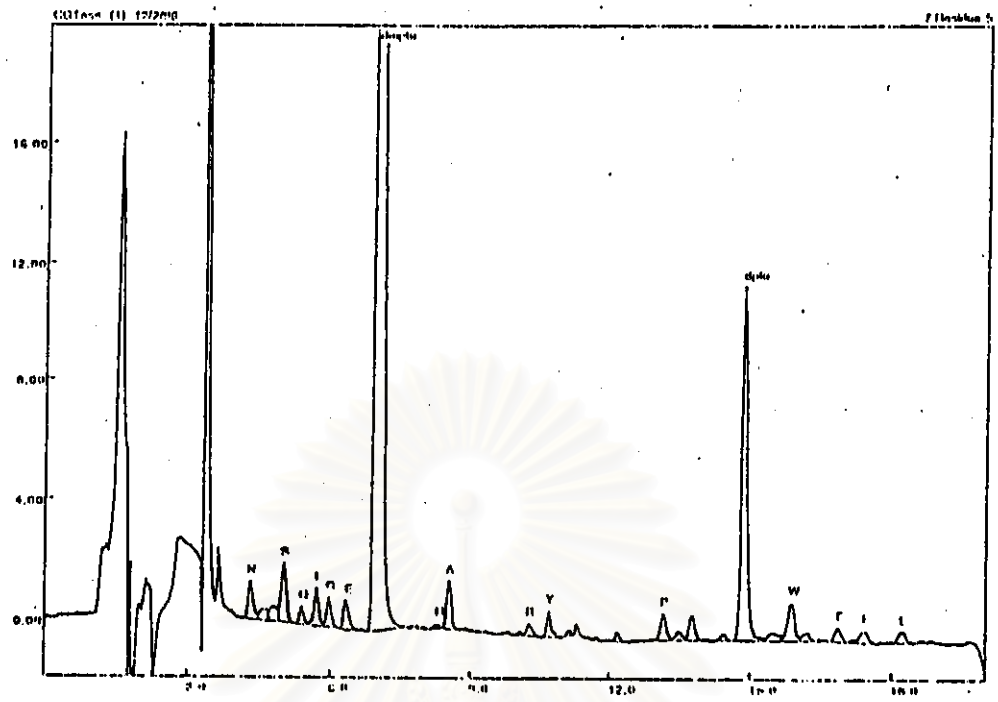


(A)

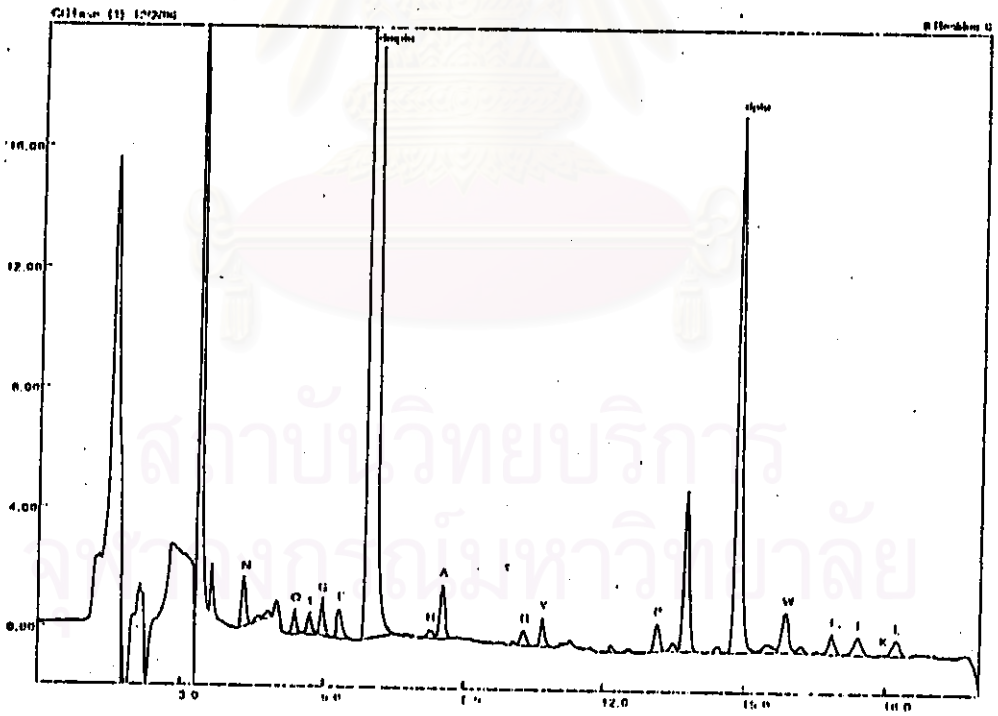


(B)

Figure 16d Chromatogram of N-terminal amino acid of purified CGTase from PTH-C18 reverse phase cartridge column
(A) Cycle 3 (B) Cycle 4

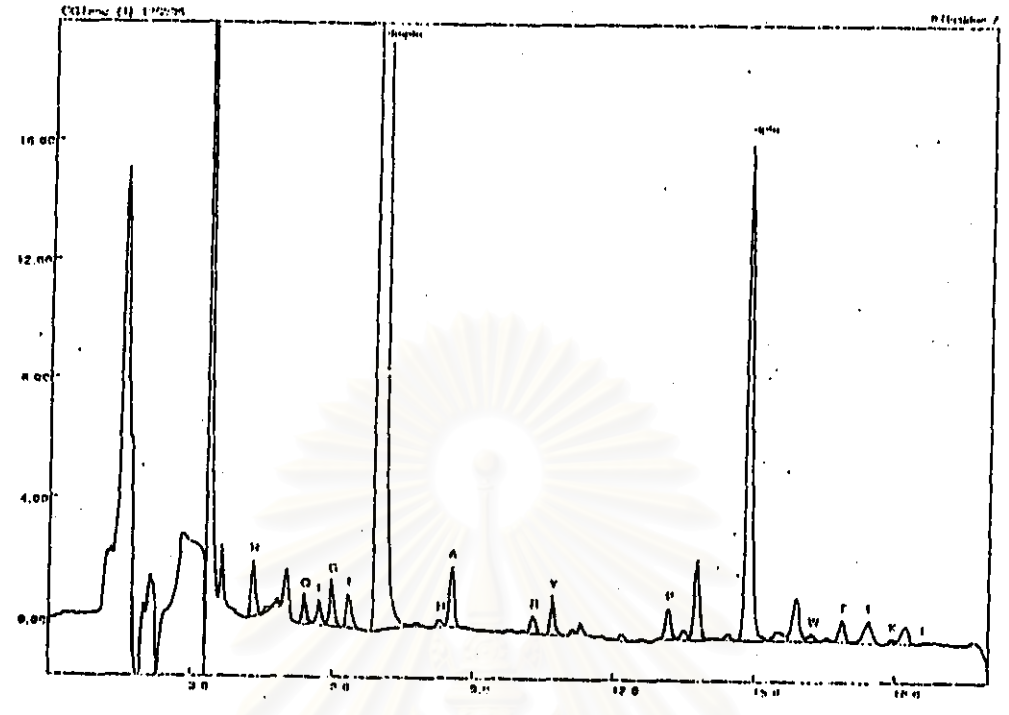


(A)



(B)

Figure 16e Chromatogram of N-terminal amino acid of purified CGTase from PTH-C18 reverse phase cartridge column
(A) Cycle 5 (B) Cycle 6



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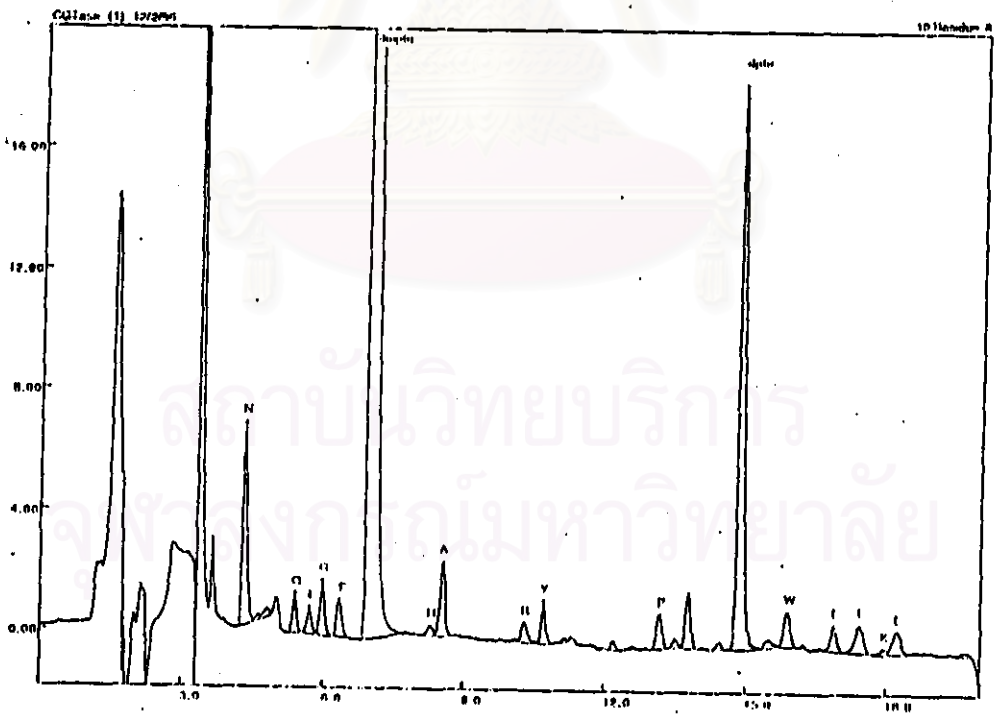
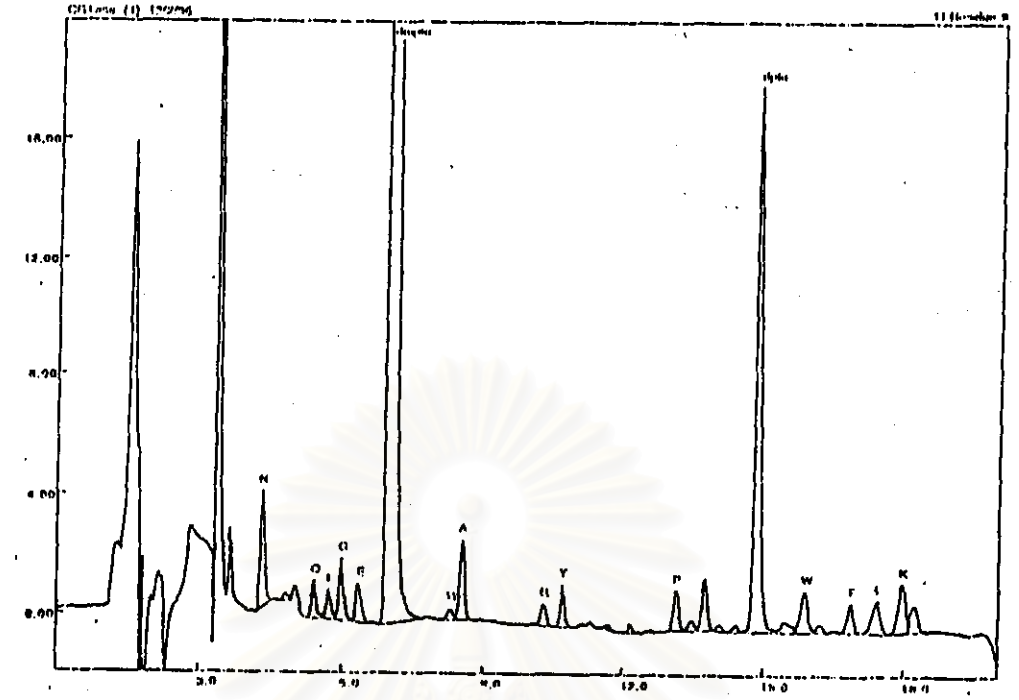
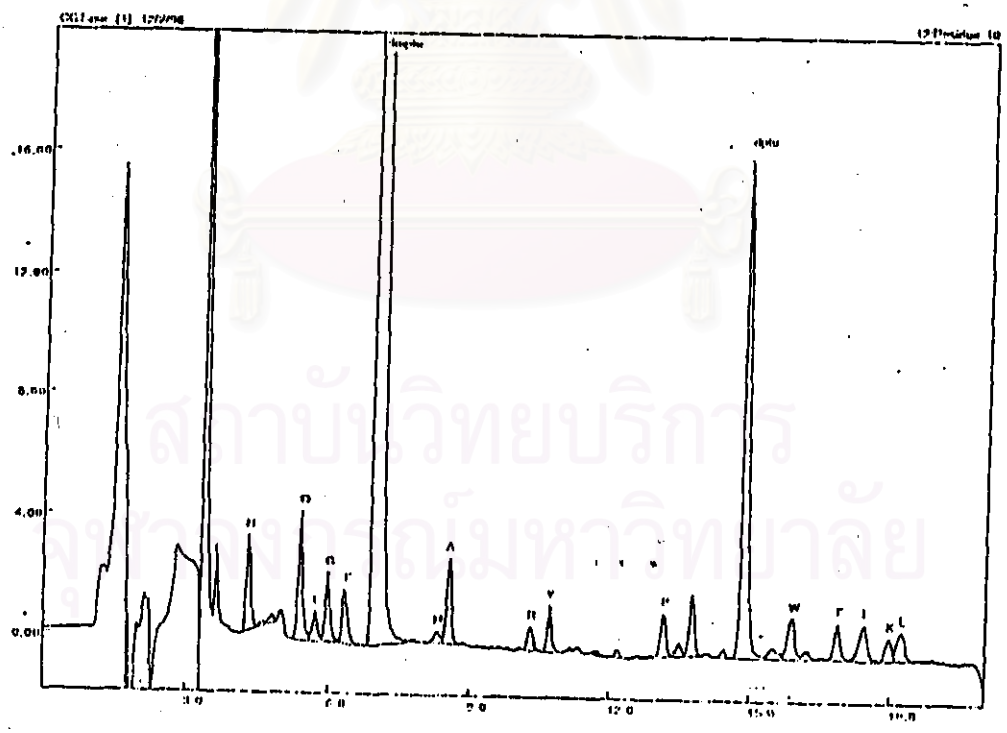


(B)

Figure 16f Chromatogram of N-terminal amino acid of purified CGTase from PTH-C18 reverse phase cartridge column
(A) Cycle 7 (B) Cycle 8

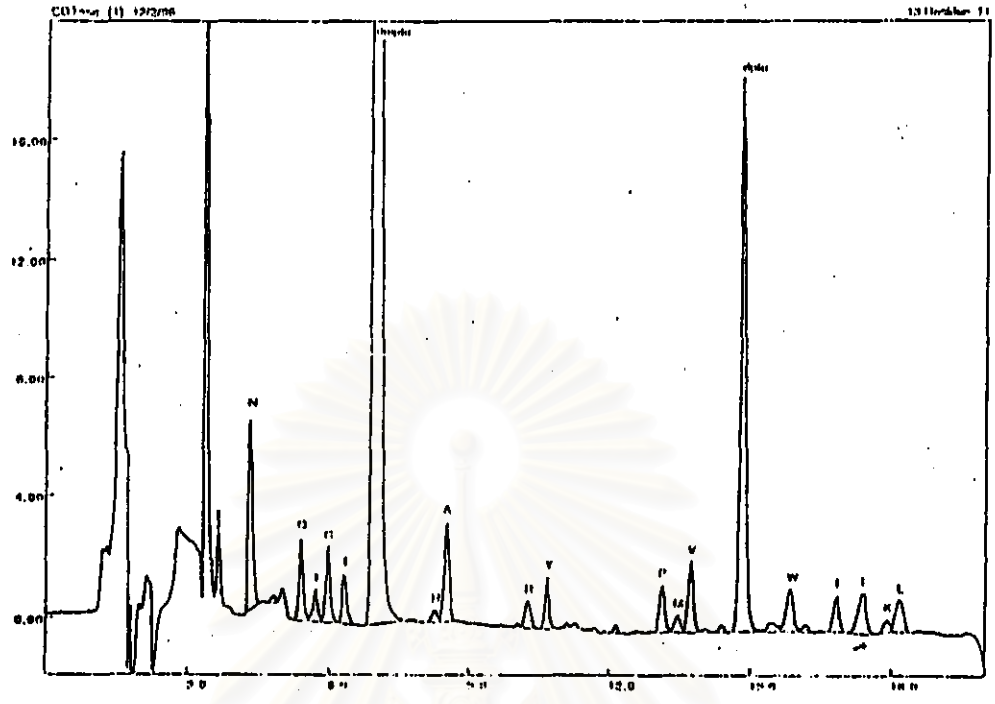


(A)

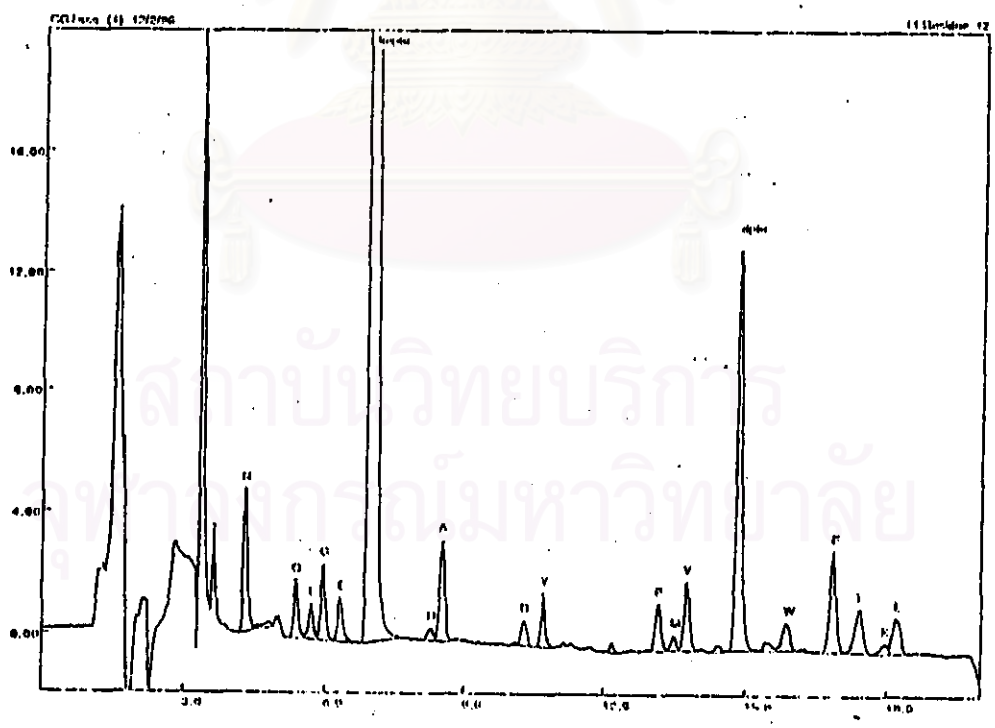


(B)

Figure 16g Chromatogram of N-terminal amino acid of purified CGTase from PTH-C18 reverse phase cartridge column
(A) Cycle 9 (B) Cycle 10

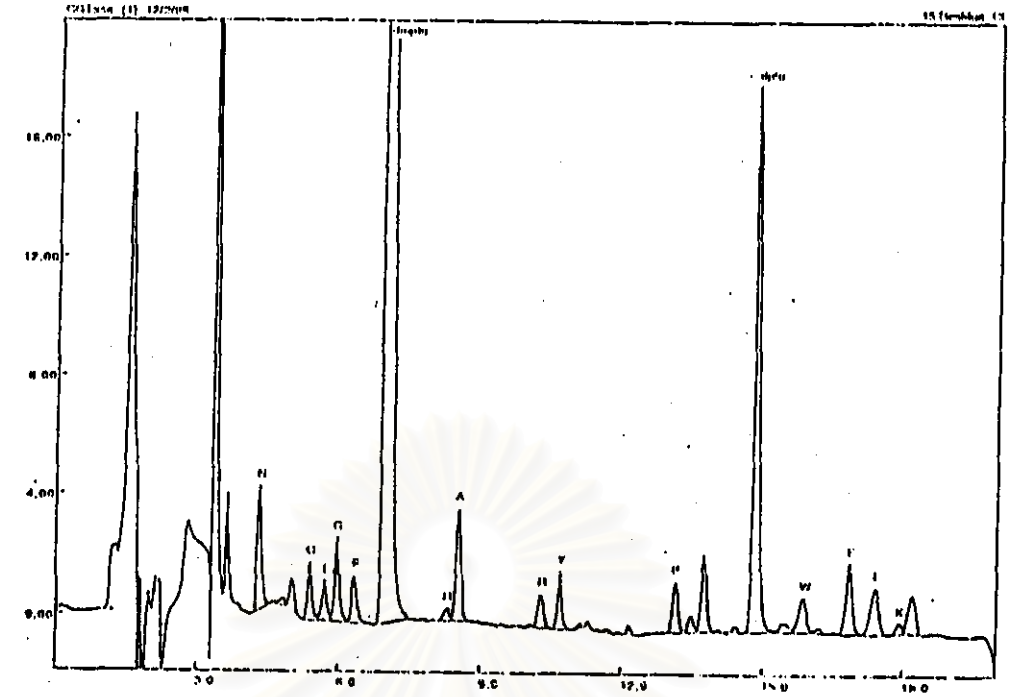


(A)

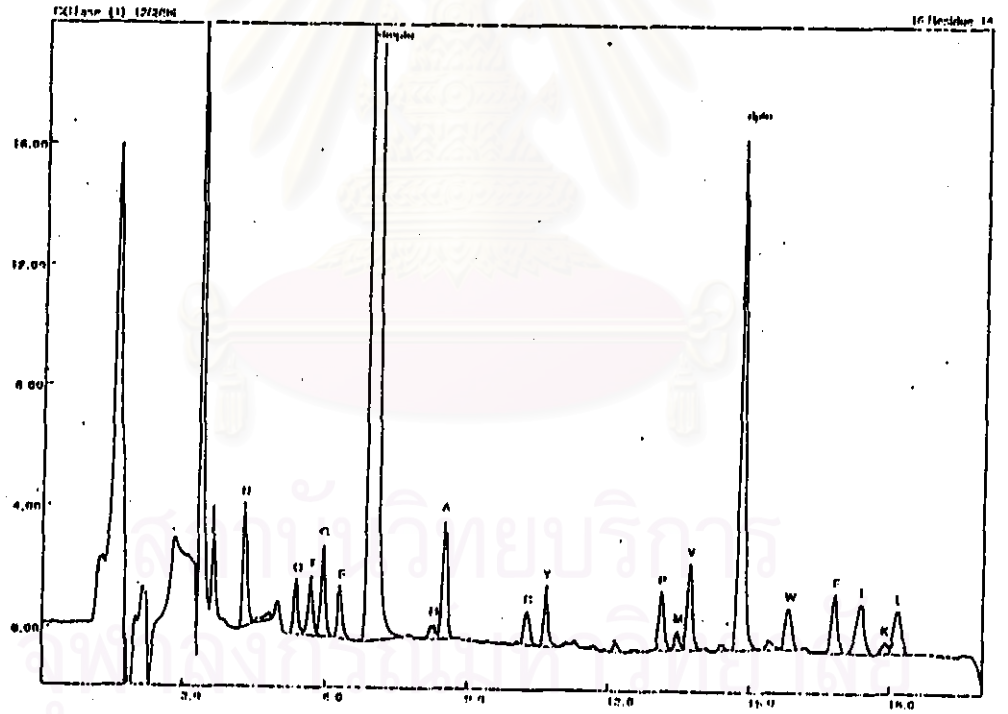


(B)

Figure 16h Chromatogram of N-terminal amino acid of purified CGTase from PTH-C18 reverse phase cartridge column
(A) Cycle 11 (B) Cycle 12

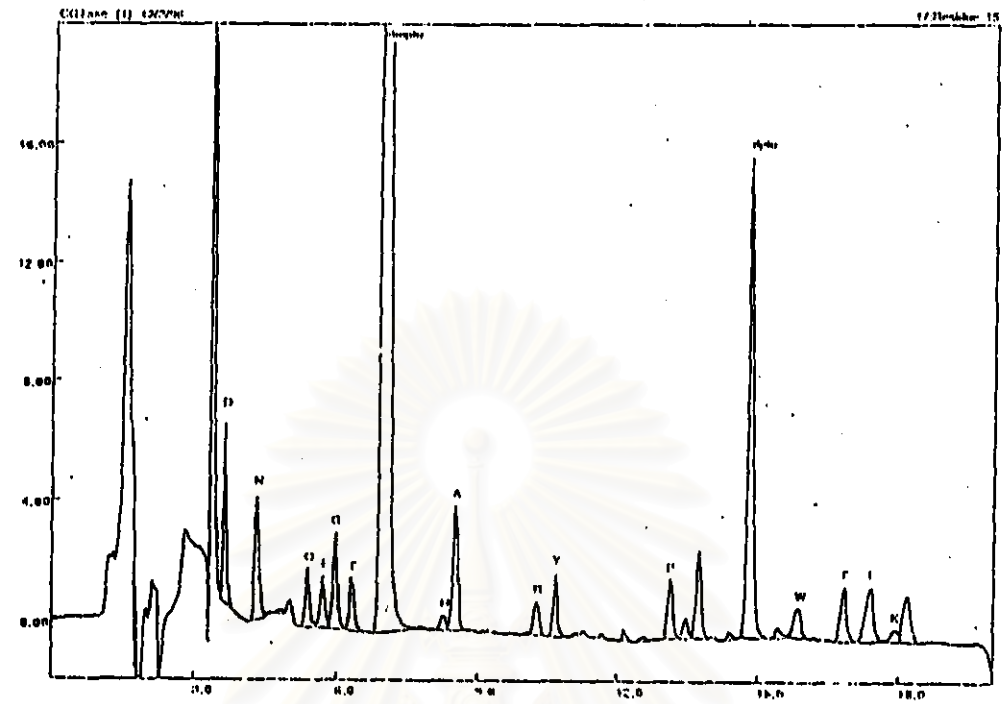


(A)

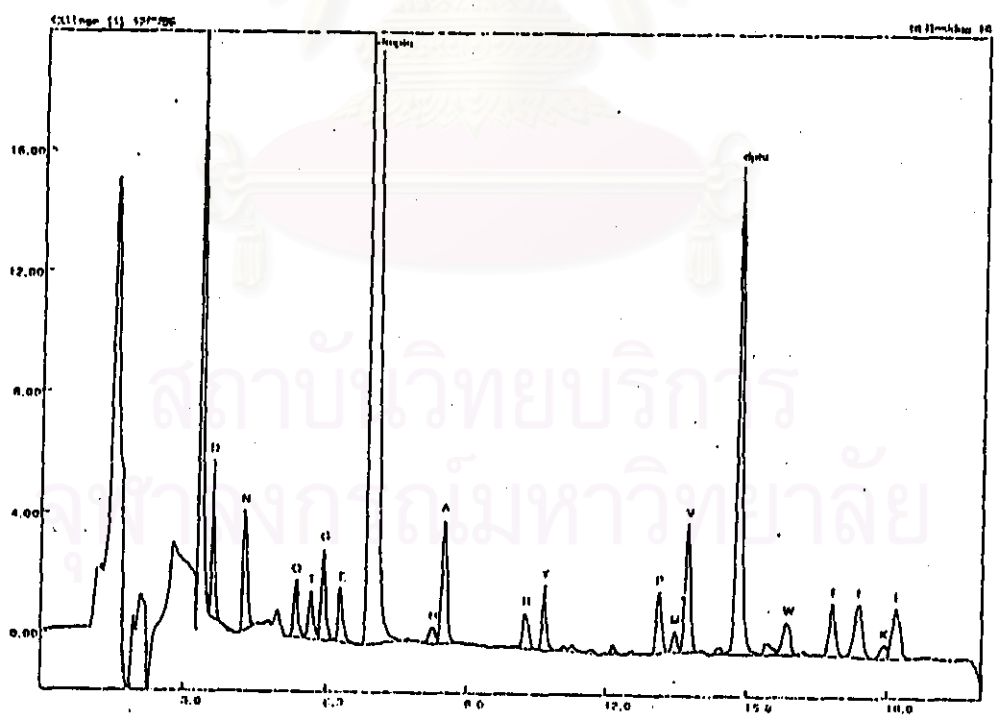


(B)

Figure 16i Chromatogram of N-terminal amino acid of purified CGTase from PTH-C18 reverse phase cartridge column
(A) Cycle 13 (B) Cycle 14

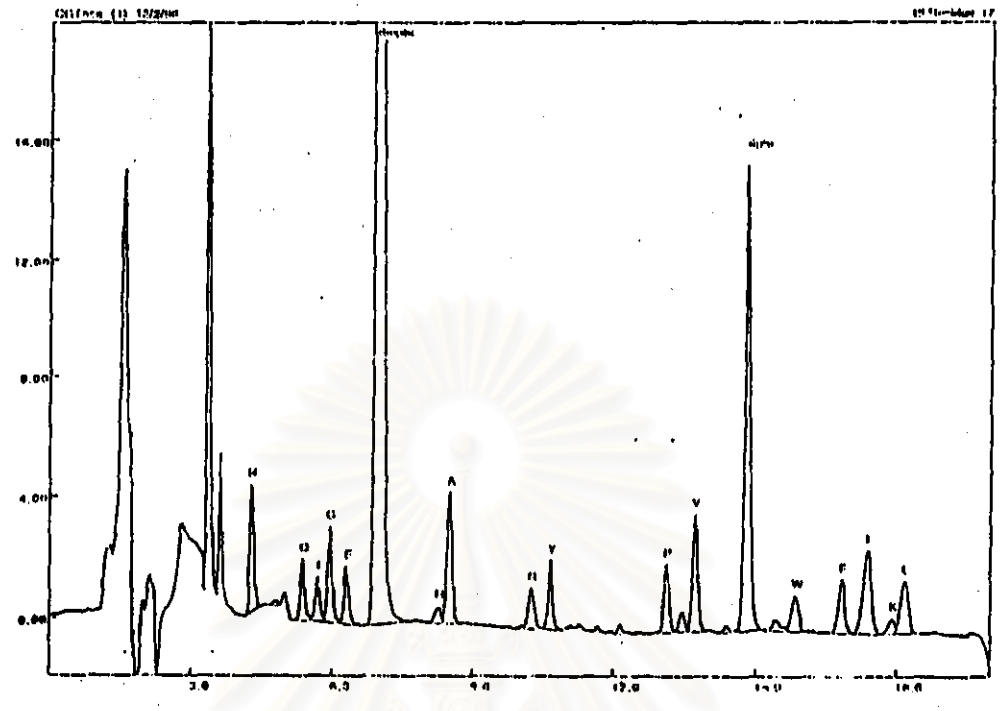


(A)

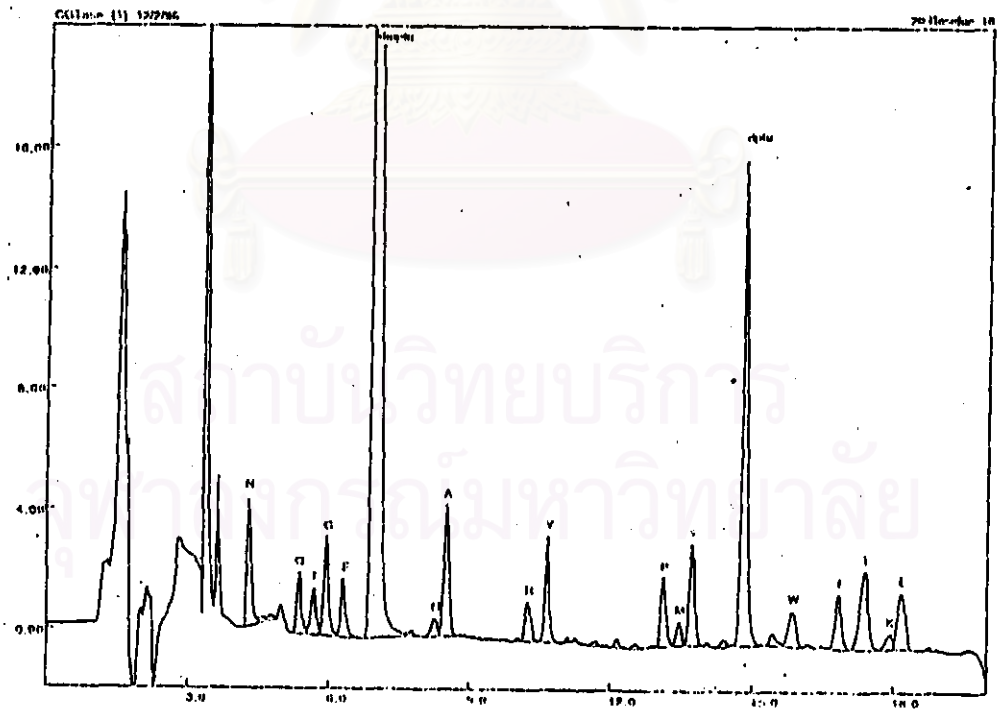


(B)

Figure 16J Chromatogram of N-terminal amino acid of purified CGTase from PTH-C18 reverse phase cartridge column
(A) Cycle 15 (B) Cycle 16

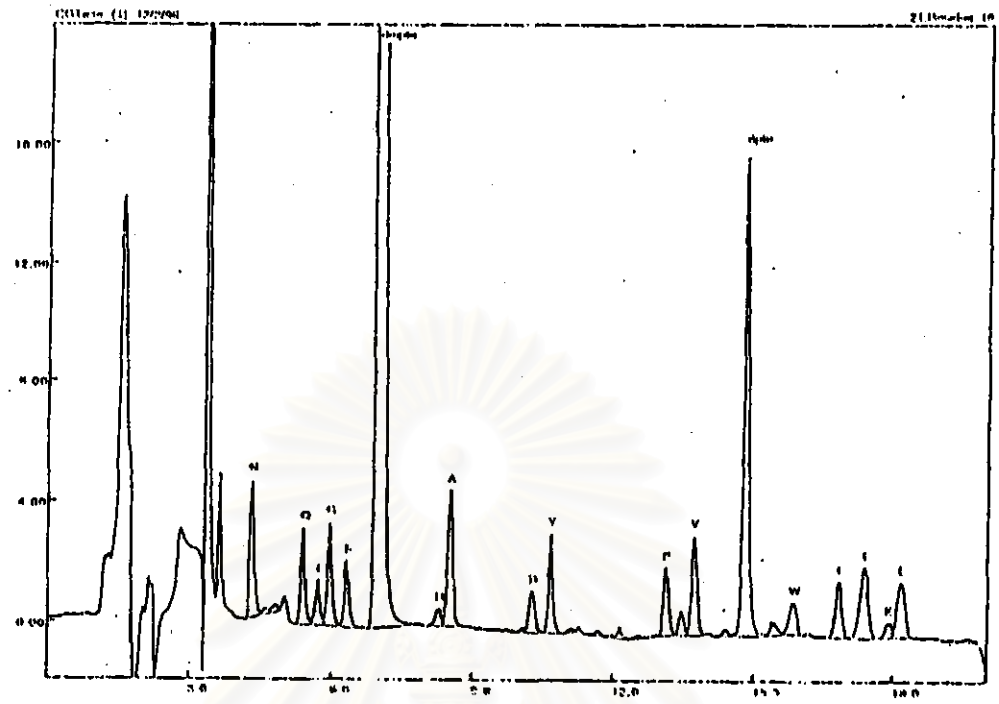


(A)

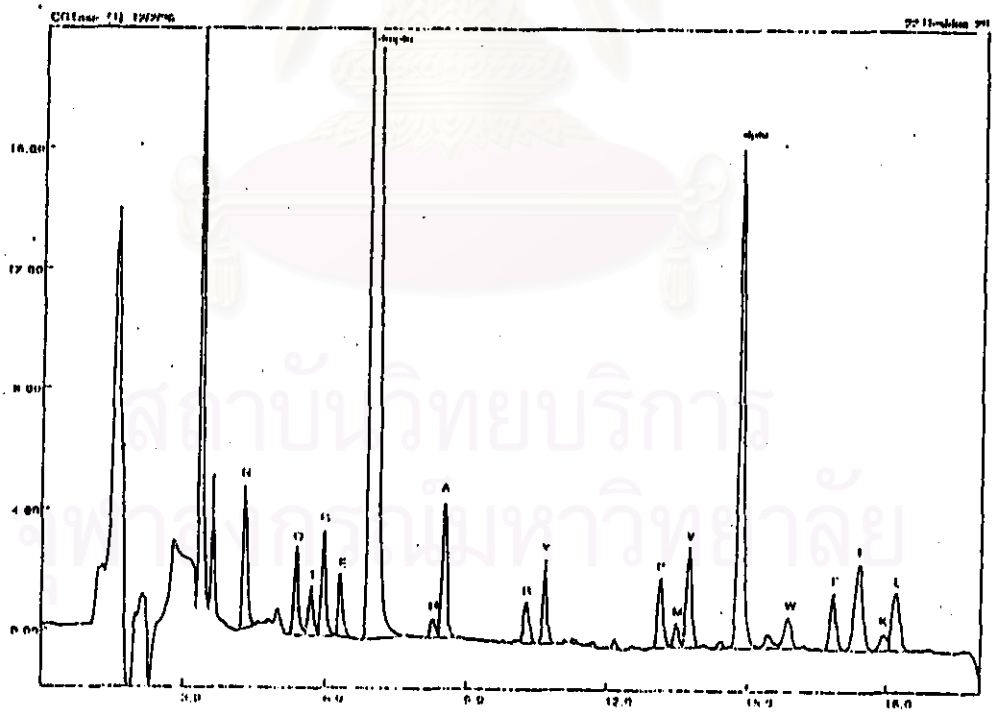


(B)

Figure 16k Chromatogram of N-terminal amino acid of purified CGTase from PTH-C18 reverse phase cartridge column
(A) Cycle 17 (B) Cycle 18



(A)



(B)

Figure 16l Chromatogram of N-terminal amino acid of purified CGTase from PTH-C18 reverse phase cartridge column
(A) Cycle 19 (B) Cycle 20

```
BA11    APDTSNY NKQNFRTDVIYQ I
1011    APDTSVS NKQNFSPDVIYQ IFTDRFSDGNPANNPTGAAFDGSCTNLRLYCGGDWQGIINKINDGY
38-2    APDTSVS NKQNFSPDVIYQ IFTDRFSDGNPANNPTGAAFDGSCTNLRLYCGGDWQGIINKINDGY
17-2    APDTSVS NKQNFSPDVIYQ IFTDRFSDGNPANNPTGAAFDGTCTNLRLYCGGDWQGIINKINDGY
8       DPDTAVT NKQSFSPDVIYQ VFTDRFLDGNPSNNPTGAAYDATCSNLRLYCGGDWQGIINKINDNY
E192    DPDTAVT NKQSFSPDVIYQ VFTDRFLDGNPSNNPTGAAYDATCSNLRLYCGGDWQGIINKINDNY
LIC     DADTAVT NKQNFSPDVIYQ IFTDRFLDGNPSNNPTGAAYDATCSNLRLYCGGDWQGIINKINDNY
MCR     SPDTSVD NKQNFSPDVIYQ IVTDRFADGDRTNNPAGDAFSGDRSNLKLYFGGDWQGIIDKINDGY
OBS     AAQADVT NKVNYTRDVIYQ IVTDRFSDGDPSNNPTGAIYSQDCSDLHKYCGGDWQGIIDKINDGY
STE     VEAAGNL NKVNFTSDVVYQ IVVDRFVDGNTSNNPSGALFSSGCTNLRKYCGGDWQGIINKINDGY

1011    LTG MGITAIWISQPVENIYSVINY-SG VNNTAYHGYWARDFKKTNPAYGTMQDFKNLIDTAHAHN
38-2    LTG MGITAIWISQPVENIYSVINY-SG VHNTAYHGYWARDFKKTNPAYGTMQDFKNLIDTAHAHN
17-2    LTG MGITAIWISQPVENIYSIINY-SG VNNTAYHGYWARDFKKTNPAYGTIADFQNLIAAAHAKN
8       FSD LGITAIWISQPVENIFATINY-SG VTNTAYHGYWARDFKKTNPYFGTMADFQNLITTAHAKG
E192    FSD LGITAIWISQPVENIFATINY-SG VTNTAYHGYWARDFKKTNPYFGTMADFQNLITTAHAKG
LIC     FSD LGITAIWISQPVENIFATINY-GG VINTAYHGYWARDFKKTNPYFGTMADFQNLISTAHAKG
MCR     LTG MGITAIWISQPVENITSVIKY-SG VNNTAYHGYWARDFKKTNDAFGDFADFQNLIDTAHAHN
OBS     LTG LGITAIWISQPVENVVALHP--SG Y--TSYHGYWARDYKRTNPFYGDFSDFDRLMDTAHSNG
STE     LTG MGVTAIWISQPVENVFSVMNDASG S--ASYHGYWARDFKKPNDFFGTLSDFQRLVDAAHAKG

1011    IKVIIDFAPNHTSPASSDDPSFAENGRLYDNGNLLGGYTNDTQN--- LFHHYGGTDFSTIENGIY
38-2    IKVIIDFAPNHTSPASSDDPSFAENGRLYDNGNLLGGYTNDTQN--- LFHHYGGTDFSTIENGIY
17-2    IKVIIDFAPNHTSPASSDQPSFAENGRLYDNGTLLGGYTNDTQN--- LFHHNGGTDFSTTENGIY
8       IKIVIDFAPNHTSPAMETDTSFAENGRLYDNGTLVGGYTNDTNG--- LFHHNGGSDFSSLENGIY
E192    IKIIIDFAPNHTSPAMETDTSFAENGKLYDNGTLVGGYTNDTNG--- LFHHNGGSDFSSLENGIY
LIC     IKIVIDFAPNHTSPAMETDTSFAENGKLYDNGNLVGGYTNDTNG--- LFHHNGGSDFSSLENGIY
MCR     IKVVIDFAPNHTSPADRDNPGFAENGGMYDNGSLLGAYSNDTAG--- LFHHNGGTDFSTIEDGIY
OBS     IKVIMDFTPNHSSPALETDPSYAENGAVYNDGVLIGNYSNDP-NN-- LFHHNGGTDFSSYEDSIY
STE     IKVIIDFAPNHTSPASETNPSYMENGRLYDNGTLLGGYTND--ANM- YFHHNGGTTFSSLEDGIY

1011    KNLYDLADLNHNNSSVDVYLKDAIKMWL DLGVDGIRVDAVKHMPFGWQKSFMATINNY KPVF
38-2    KNLYDLADLNHNNSSVDVYLKDAIKMWL DLGVDGIRVDAVKHMPFGWQKSFMATINNY KPVF
17-2    KNLYDLADLNHNNSTVDVYLKDAIKMWL DLGVDGIRMDAVKHMPFGWQKSFMAAVNNY KPVF
8       KNLYDLADFNHNNATIDKYFKDAIKLWL DMGVDGIRVDAVKHMPLGWQKSWMSSIYAH KPVF
E192    KNLYDLADFNHNNATIDKYFKDAIKLWL DMGVDGIRVDAVKHIALGWQKSWMSSIYVH KPVF
LIC     KNLYDLADLNHNNSTIDQYFKDAIKLWL DMGVDGIRVDAVKHMPLGWQKSWMSSIYAH KPVF
MCR     KNLYDLADINHNNNAMDAYFKSAIDLWL GMGVDGIRFDAVKHMPFGWQKSFVSSIYGGDHPVF
OBS     RNLYDLADYDLNNTVMTQYLKESIKLWL DKGIDGIRVDAVKHMSEGWQTSLMSDIYAH EPVF
STE     RNLFDLADLNHQNPVIDRYLKDAVKMWI DMGIDGIRMDAVKHMPFGWQKSLMDEIDNY RPVF

1011    TFGEWFLGVNE ISPEYHQFANESGMSLLDFRFAQKARQVFRDNTDNMYGLKAMLEGSE VDYAQ
38-2    NFGEWFLGVNE ISPEYHQFANESGMSLLDFPFAQKARQVFRDNTDNMYGLKAMLEGSE VDYAQ
17-2    TFGEWFLGVNE VSPENHQFANESGMSLLDFRFAQKVRQVFRDNTDNMYGLKAMLEGSA ADYAQ
8       TFGEWFLGSAA SDADNTDFANKSGMSLLDFRFNSAVRNVFRDNTSNMYALDSMINSTA TDYNQ
E192    TFGEWFLGSAA SDADNTDFANKSGMSLLDFRFNSAVRNVFRDNTSNMYALDSMINSTA TDYNQ
LIC     TFGEWFLGSAA SDADNTEFANESGMSLLDFRFNSAVRDVFRDNTSNMYALDSMITGTA ADYNQ
MCR     TFGEWYLGADQ TDGDNIKFANESGMNLLDFEYAQEVREVFRDKTETMKDLYEVLASTE SQYDY
OBS     TFGEWFLGSGE VDPQNHHFANESGMSLLDFQFGQTIRDVLMDGSSNWYDFNEMIASTE EDYED
STE     TFGEWFLSENE VDANNHYFANESGMSLLDFRFGQKLRQVLRNNSDNWYGFNQMIQDTA SAYDE

1011    VNDQVTFIDNHDMERFHTSNGDRRKLEQALAFTLTSRGVPAIYYGSEQYMSGGNDPDNRARLP SFST
38-2    VNDQVTFIDNHDMERFHTSNGDRRKLEQALAFTLTSRGVPAIYYGSEQYMSGGNDPDNRARIP SFST
17-2    VNDQVTFIDNHDMERFHASNANRRKLEQALAFTLTSRGVPAIYYGTEQYMSGGTDPDNRARIP SFST
8       VNDQVTFIDNHDMDRFKTSAVNNRKLEQALAFTLTSRGVPAIYYGTEQYLTGNGDPDNRAKMP SFSK
E192    VNDQVTFIDNHDMDRFKTSAVNNRKLEQALAFTLTSRGVPAIYYGTEQYLTGNGDPDNRAKMP SFSK
LIC     VNDQVTFIDNHDMDRFKTSAVNNRKLEQALAFTLTSRGVPAIYYGTEQYLRGNGDPDNRAKMP SFSK
MCR     INNMVTFIDNHDMSRFSVGSSSTRATEQALAFTLTSRGVPAIYYGTEQYMTGDGDPNNRAMMT SFNT
OBS     VNDQVTFIDNHDMDRFMIDGGDNRHTDIALAFTLTSRGVPTIYYGTEQYLTGGNDPENRKPMS DFDR
STE     VNDQVTFIDNHDMDRFMIDGGDPRKVDMALAVLLTSRGVPNIYYGTEQYMTGNGDPNNRKMMS SFNK
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1011    TTTAYQVIQKLAPLRKSNPAIAYGSTHERWINNDVIIYERKFGNNVAVVAINRNTMNT PASITGLV
38-2    TTTAYQVIQKLAPLRKSNPAIAYGSTQERWINNDVIIYERKFGNNVAVVAINRN MNT PASITGLV
17-2    STTAYQVIQKLAPLRKCNPAIAYGSTQERWINNDVLIYERKFGSNVAVVAVNRNL NA PASISGLV
8       STTAFNVISKLAPLRKSNPAIAYGSTQQRWINNDVYVYERKFGKSVAVVAVNRNL ST SASITGLS
E192    STTAFNVISKLAPLRKSNPAIAYGSTQQRWINNDVYVYERKFGKSVAVVAVNRNL ST PANITGLS
LIC     TTTAFNVISKLAPLRKSNPAIAYASTQQRWINNDVYIYERKFGKSVAVVAVNRNL ST PASIANLS
MCR     GTTAYKVIQALAPLRKSNPAIAYGTTTERWVNNDVLIIERKFGSSAALVAINRN SSA AYPISGLL
OBS     TTNSYQIISTLASLRQNNPALGYGNTSERWINSDVYIYERSFGDSVVLTAVN  SGDT SYTINNLN
STE     NTRAYQVIQKLSSLRRNNPALAYGDTEQRWINGDVYVYERQFGKDVVLVAVNR SSSS NYSITGLF

1011    TSLRRASYNDVLGGILNGN LTVGAGGAASNFTLAPGGTAVWQYTTDATTPIIG NVGPMMAKPGVT
38-2    TSLPQGSYNDVLGGILNGNTLTVGAGGAASNFTLAPGGTAVWQYTTDATAPING NVGPMMAKAGVT
17-2    TSLPQGSYNDVLGGLLNGNTLSVGAGGAASNFTLAAGGTAVWQYTAATATPTIG HVGPMMAKPGVT
8       TSLPTGSYNDVLGGVLNGNNIT STNGSINNFTLAAGATAVWQYTTAETTPTIG HVGPVMGKPGNV
E192    TSLPTGSYNDVLGGVLNGNNIT SSNGSVNSFTLAAGATAVWQYTAAETTPTIG HVGPVMGKPGNV
LIC     TSLPTGNYNDVLGGVLNGNNIT STNGNVSSFTLAAGATAVWQYTTSETTPTIG HVGPVMGKPGNV
MCR     SSLPAGTYSDVLNGLLNGNSITVGSGGAVTNFTLAAGGTAVWQYTAPETSPAIG NVGPTMGQPGNV
OBS     TSLPQGQYTDELQQLLDGNEITVNSNGAVDSFQLSANGVSVWQITEEHASPLIG HVGPMMGKHGNT
STE     TALPAGTYTDQLGGLLDGNTIQVGSNGSVNAFDLGPGEVGVWAYSATESTPIIG HVGPMMGQVGHQ

1011    ITIDGRGFGSGKGTVYFGTTAVTGADIVAWEDTQIQVKIPAVPGGIYDI RVANAAGAASNIYDNFEV
38-2    ITIDGRASG RQGTVYFGTTAVTGADIVAWEDTQIQVKILRVPGGIYDI RVANAAGAASNIYDNFEV
17-2    ITIDGRGFGSSKGTVYFGTTAVSGADITSWEDTQIKVKIPAVAGGNYNI KVANAAGTASNVYDNFEV
8       VTIDGRGFGSTKGTVYFGTTAVTGAAITSWEDTQIKVTIPSVAAGNYAV KVA-ASGVNSNAYNNFTI
E192    VTIDGRGFGSTKGTVYFGTTAVTGAAITSWEDTQIKVTIPSVAAGNYAV KVA-ANGVNSNAYNNFTI
LIC     VTISGRGFGSTKGTVYFGTTAVTGAAITSWEDTQIKVTIPAVAAGNYAV KVA-ANGVNSNAYNNFTI
MCR     VTIDGRGFGGTAGTVYFGTTAVTGSGIVSWEDTQIKAVIPKVAAGKTGV SVKTSSGTASNTFKSFNV
OBS     VTITGEGFGDNEGSVLFDSD---FSDVISWSDTKIEVSVPDVTAGHYDI SVVNAGDSQSPTYDKFEV
STE     VTIDGEGFGTNTGTVKFGTT---AANVVSWSNNQIVVAVPNVSPGKYNI TVQSSSGQTSAAYDNFEV

1011    LTGDQV TVRFVINNATTALGQNVFLTGNVSELGNWD--P-NNAIG PMYNQVV
38-2    LTGDQV TVRFVINNATTALGQNVFLTGNVSELGNWD--P-NNAIG PMYNQVV
17-2    LTGDQV SVRFVVNNATTALGQNVYLTGSVSELGNWD--PA-KAIG PMYNQVV
8       LTGDQV TVRFVVNNASTTLGQNLYLTGNVAELGNW-STG-STAIG PAFNQVI
E192    LTGDQV TVRFVINNASTTLGQNIYLTGNVAELGNW-STG-STAIG PAFNQVI
LIC     LTGDQV TVRFVINNASTTLGQNIYLTGNVAELGNW-STG-TTAIG PAFNQVI
MCR     LTGDQV TVRFLVNQANTNYGTNVYLVGNAAELGSWD--P-NKAIG PMYNQVI
OBS     LTGDQV SIRFAVNNATTSLGTNLYMVGNVNELGNWD--PD QAIG PMFNQVM
STE     LTNDQV SVRFVVNNATTNLGQNIYIVGNVYELGNWDTS---KAIG PMFNQVV

1011    YQ YPTWYYDVSVPAGQTI EFKFLKKQGS-TVTWEGGANRTFTTPTSG TATVNVNWQP
38-2    YQ YPTWYYDVSVPAGQTI EFKFLKKQGS-TVTWEGGANRTFTTPTSG TATVNVNWQP
17-2    YQ YPNWYYDVSVPAGKTI EFKFLKKQGS-TVTWEGGSNHTFTAPSSG TATINVNWQP
8       HQ YPTWYYDVSVPAGKQL EFKFLKKNGS-TITWEGGSNHTFTTPASG TATVNVTWQ
E192    HQ YPTWYYDVSVPAGKEL EFKFLKKNGS-TITWEGGSNHKFTTPASG TATVNVTWQ
LIC     HT YPTWYYDVSVPAGKQL EFKFLKKNGS-TVTWEGGGNHTYTTPASG TATVNVNWQ
MCR     A- YPSWYYDVSVPAGTKL DFKFFKKNGA-TITWEGGSNHTFTTPTRG VGTVTVDWQN
OBS     YQ YPTWYYDISVPAEENL EYKFIKKDSSGNVVWESGNNHTYTTPATG TDTVLVDWQ
STE     YS YPTWYIDVSVPEGKTI EFKFIKKDSQGNVTWESGSNHVYTTPTNT TGKIIVDWQN
```

Figure 17 Comparison of 20 residues of N-terminal amino acid sequence of CGTase from *Bacillus* sp.A11 with whole amino acid of CGTases from other bacterial strains (BA11, *B.*sp. A11; 1011, *B.*sp. No.1011; 17-2, *B.*sp. No.17-2; 8, *B.* circulans No. 8; E192, *B. circulans* E192; LIC, *B. licheniformis*; MCR, *B. macerans*; OBS, *B.ohbensis*; STE, *stearothermophilus*). Homologous sequences, used to design the synthetic oligonucleotides, were surrounded by rectangles.

BA11	A	P	D	T	S	N	Y	N	K	Q	N	F	R	T	D	V	I	Y	Q	I
1011	A	P	D	T	S	V	S	N	K	Q	N	F	S	T	D	V	I	Y	Q	I
38-2	A	P	D	T	S	V	S	N	K	Q	N	F	S	T	D	V	I	Y	Q	I
17-2	A	P	D	T	S	V	S	N	K	Q	N	F	S	T	D	V	I	Y	Q	I
8	D	P	D	T	A	V	T	N	K	Q	S	F	S	P	D	V	I	Y	Q	V
E192	D	P	D	T	A	V	T	N	K	Q	S	F	S	P	D	V	I	Y	Q	V
LIC	D	A	D	T	A	V	T	N	K	Q	N	F	S	P	D	V	I	Y	Q	I
MCR	S	P	D	T	S	V	D	N	K	V	N	F	S	P	D	V	I	Y	Q	I
OBS	A	A	Q	A	D	V	T	N	K	V	N	Y	T	R	D	V	I	Y	Q	I
STE	V	E	A	A	G	N	L	N	K	V	N	F	T	S	D	V	V	Y	Q	I

(A)

TA	C	AAC	AA(G/T)	CAG	AAT	TTC	C G(C/T)
TA	C	AAC	AA(G/T)	CAG	AAT	TTC	C GC
TA	C	AAC	AA(G/T)	CAG	AAT	TTC	C GC
TA	C	AAC	AA(G/T)	CAG	AAT	TTC	C GC
AC	C	AAC	AAA	CAG	AGC	TTC	A GT
AC	C	AAC	AAA	CAG	AGC	TTC	A GT
AC	C	AAC	AAA	CAG	AAT	TTC	A GC
GA	C	AAC	AAG	GTC	AGT	TTC	A GT
AC	C	AAC	AAG	GTC	AAT	TAC	A CC
TT	C	AAC	AAG	GTC	AGT	TTC	A GT

(B)

PNB = C AAC AAG CA(G/C) AAT TTC C

(C)

Figure 18 N-terminal amino acid sequences of CGTases (A) from BA11 (*B.*sp.A11), 1011 (*Bacillus* sp. No.1011), 38-2 (*B.*sp. No.38-2), 17-2 (*B.* sp. No.17-2), 8 (*B. circulans* No.8), E192 (*B. circulans* No.E192), LIC (*B. licheniformis*), MCR (*B. macerans*), OBS (*B.ohbensis*), and STE (*B. stearothermophilus*), (B) decoded to nucleotide sequences and (C) to PNB (synthetic oligonucleotide).

1011	Y	P	T	W	Y	Y	D	V	S	V	P	A	G	Q	T	I
38-2	Y	P	T	W	Y	Y	D	V	S	V	P	A	G	Q	T	I
17-2	Y	P	N	W	Y	Y	D	V	S	V	P	A	G	K	T	I
8	Y	P	T	W	Y	Y	D	V	S	V	P	A	G	K	Q	L
E192	Y	P	T	W	Y	Y	D	V	S	V	P	A	G	K	E	L
LIC	Y	P	T	W	Y	Y	D	V	S	V	P	A	G	K	Q	L
MCR	Y	P	S	W	Y	Y	D	V	S	V	P	A	G	T	K	L
OBS	Y	P	T	W	Y	Y	D	I	S	V	P	A	E	E	N	L
STE	Y	P	T	W	Y	I	D	V	S	V	P	E	G	K	T	I

(A)

TG	G	TAT	TAT	GAT	GTC	AGC	G	TT	CCG	GCA	GGC
TG	G	TAT	TAT	GAT	GTC	AGC	G	TT	CCG	GCA	GGC
TG	G	TAT	TAT	GAT	GTC	AGC	G	TT	CCG	GCA	GGC
TG	G	TAC	TAT	GAT	GTC	AGC	G	TA	CCG	GCA	GGC
TG	G	TAC	TAT	GAT	GTC	AGC	G	TA	CCG	GCA	GGC
TG	G	TAC	TAT	GAT	GTC	AGC	G	TA	CCG	GCA	GGC
TG	G	TAT	TAT	GAT	GTC	AGC	G	TG	CCG	GCG	GGG
TG	G	TAC	TAT	GAT	ATA	AGC	G	TT	CCT	GCC	GAG
TG	G	TAC	GTC	GAT	GTC	AGC	G	TA	CCG	GAG	GGC

(B)

PCC = G TA(T/C) TAT GAT GTC AGC G

(C)

Figure 19 C-terminal amino acid sequences of CGTases (A) from 1011 (*Bacillus* sp. No.1011), 38-2 (*B.*sp. No.38-2), 17-2 (*B.* sp. No.17-2), 8 (*B. circulans* No.8), E192 (*B. circulans* No.E192), LIC (*B. licheniformis*), MCR (*B. macerans*), OBS (*B.ohbensis*), and STE (*B. stearothermophilus*). (B) Nucleotide sequences of CGTases from above strains were compared, and (C) the sequence for synthetic oligonucleotide (PCC) was designed.

amino acid sequence of CGTase from published reports were also compared as shown in Figure 17 and 19. The 16 residues C-terminal amino acid sequence with homologies of 60% amino acid identity was also chosen to be analyzed further. Then two oliginucleotides were designed by decoding the N- and C- terminal amino acid sequence to nucleotide sequences. The designed oligonucleotides from N- and C-terminal amino acid sequence were, respectively, called PNB and PCC. The sequences were

PNB; 5′-CAACAAGCA(G/C)AATTTCC-3′ and

PCC; 5′-GTA(C/T)TATGATGTCAGCG- 3′

The oligonucleotides were synthesized by Bio-synthesis, U.S.A.

DNA extraction and preparation of *Bacillus* sp. A11 for cloning

DNA was extracted from *Bacillus* sp.A11 using extraction procedure modified from Rodiquez and Tsin, 1983. The extracted DNA was shown to be high molecular weight band of greater than 23.1 kb when analyzed with agarose gel electrophoresis as shown in Figure 20. The A_{260}/ A_{280} ratio was higher than 1.8 indicating high purity of DNA. The extracted DNA was partially digested with 10 units of *Sau*3AI and incubated at 37 ^{0}C for 10, 20, 30, 40, 50, 60, 120, and 180 minutes, respectively. Smear bands between 2-6 kb at 60 minute digestion were chosen and eluted from agarose gel by electroelution. The yield of recovered DNA was estimated to be 100 ng/μl, and the DNA was used for DNA library construction.

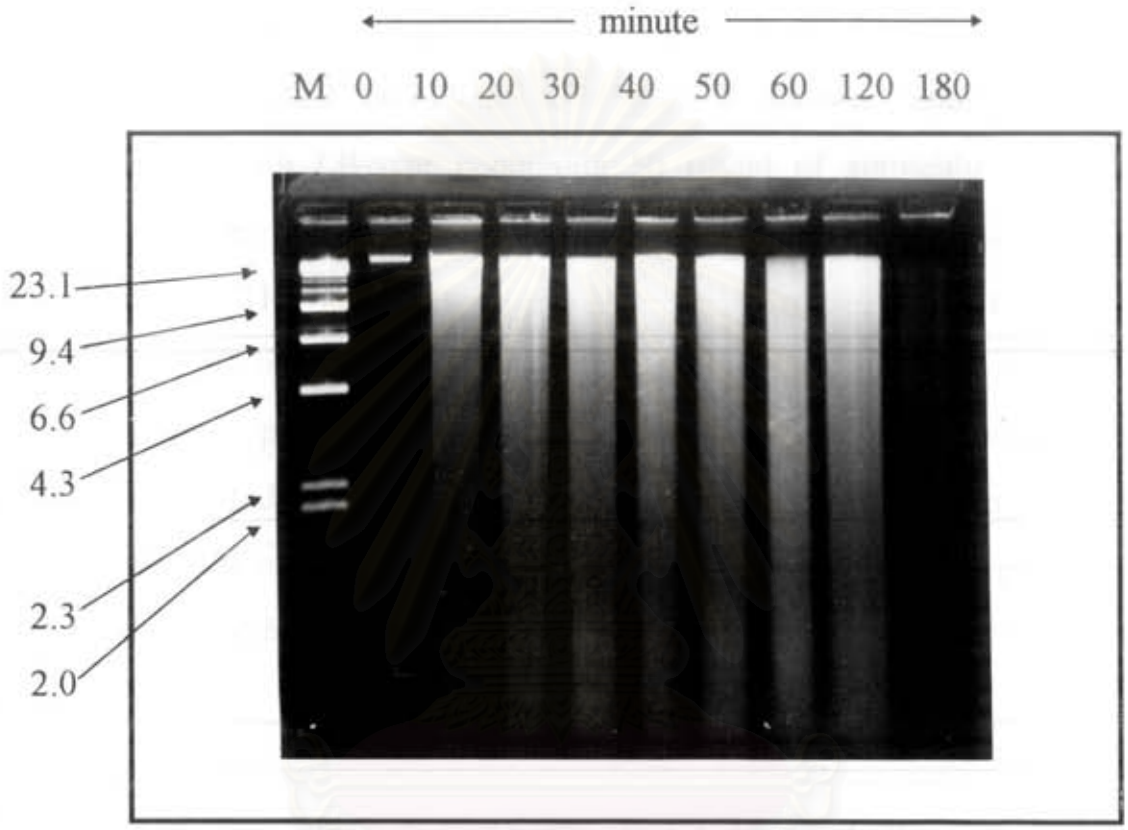


Figure 20 Analysis of partially digested chromosomal DNA from *Bacillus* sp.A11 with *Sau* 3AI on 1.0% agarose gel electrophoresis

M: Standard DNA marker (λ/*Hin*dIII)

Chromosomal DNA was partially digested with *Sau*3AI for 0, 10, 20, 30, 40, 50, 60, 120, and 180 minutes, respectively.

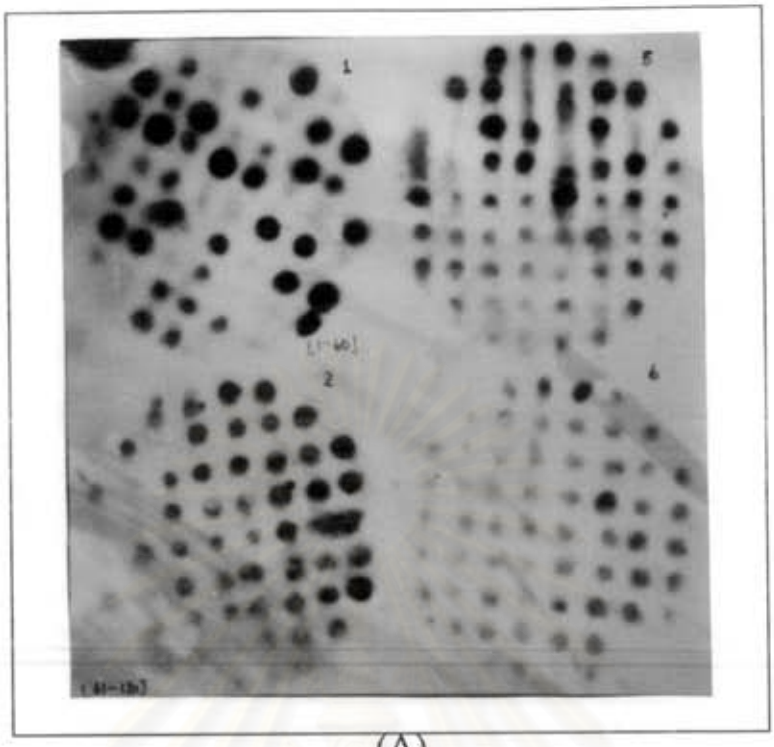
Library construction and screening

Genomic DNA fragments of 2-6 kb were ligated with *Bam*HI-digested and phosphatased pUC18 DNA vector. 5 μl of ligation mixture was transformed into *E. coli* DH5α cells by $CaCl_2$ method. Transformants were selected with LB-agar containing 50 μg/ml of ampicillin. Partial genomic libraries of *Bacillus* sp.A11 of about 1,114 transformant clones were obtained. They were lifted onto nylon membrane and screened for CGTase gene by colony hybridization with [γ-^{32}P] end labeled oliogonucleotide probes. After intensive screening, a total of about 360 clones were tested positive with both PNB and PCC probes. Examples of colony hybridization were shown in Figure 21 and 22. Individual positive clones which gave strong hybridization signal were picked up to test for CGTase activity.

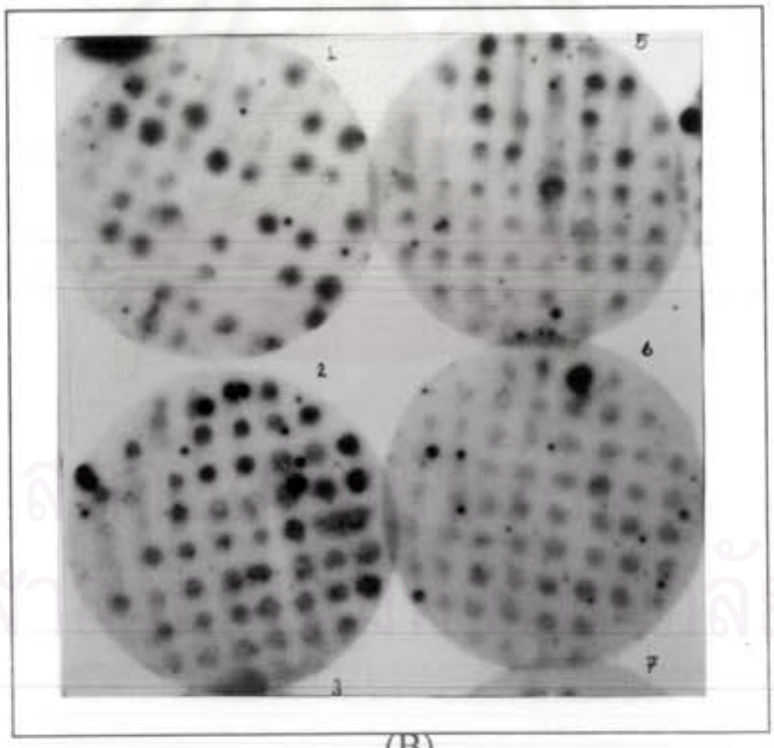
Screening of transformants containing the CGTase activity

Amylolytic activity

Transformants with strong hybridization signal were plated onto LB-starch agar, containing 50 μg/ml of ampicillin, and grown at 37^0 C for 60 hours. Iodine solution was added to the plate. Colonies with amylolytic activity would show surrounding clear zone. 20 colonies had clear zone. An example was shown in Figure 23. *E. coli* DH5α harboring pUC18 had no amylolytic activity.



(A)



(B)

Figure 21 Examples of colonies with strong hybridization signal from colony hybridization screening with the ^{32}P labeled PNB oligonucleotide probes after washing at (A) room temperature and (B) 40^0C

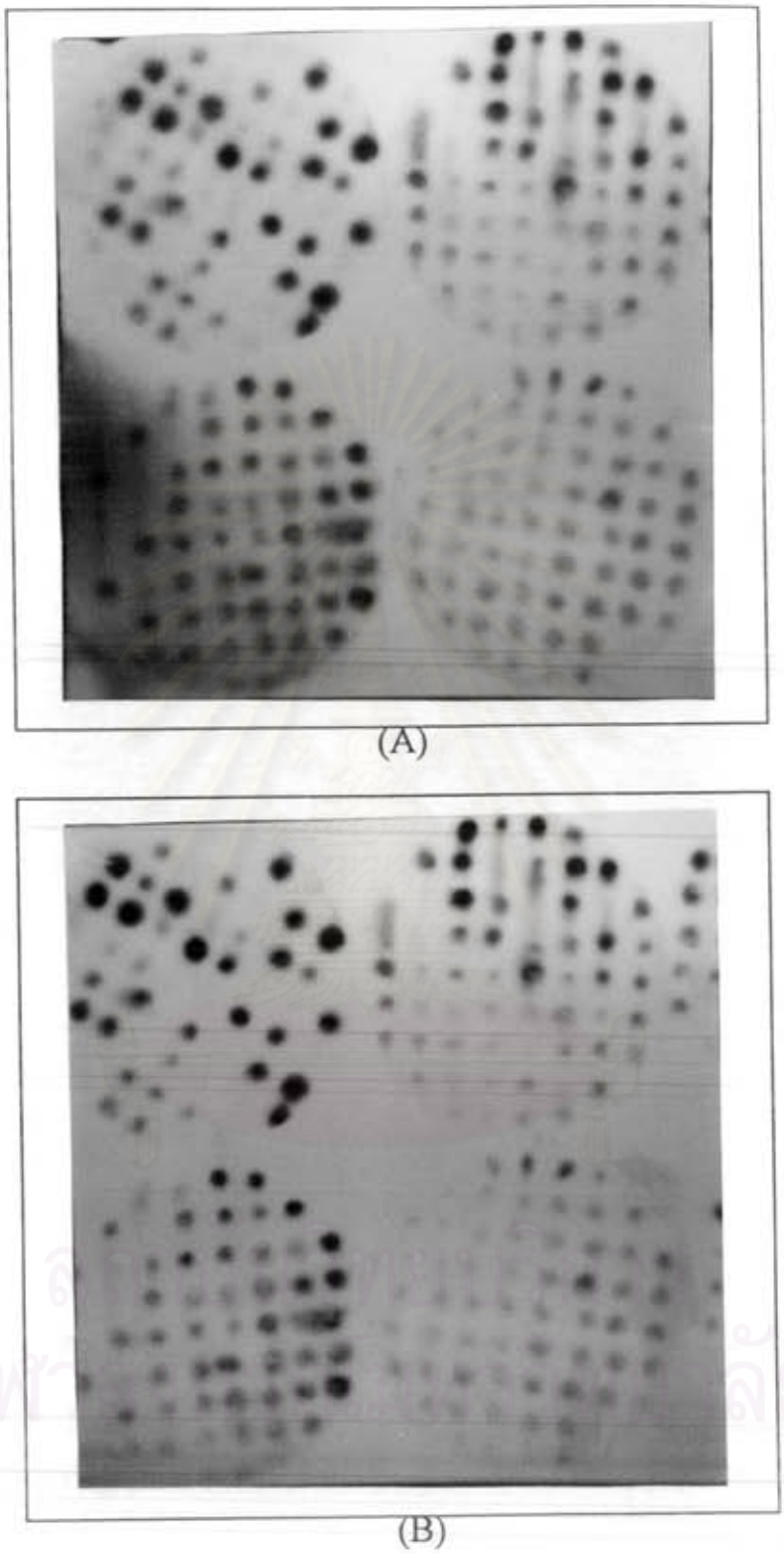


Figure 22 Examples of colonies with strong hybridization signal from colony hybridization screening with the ^{32}P labeled PCC oligonucleotide probes after washing at (A) room temperature and (B) 40^0C

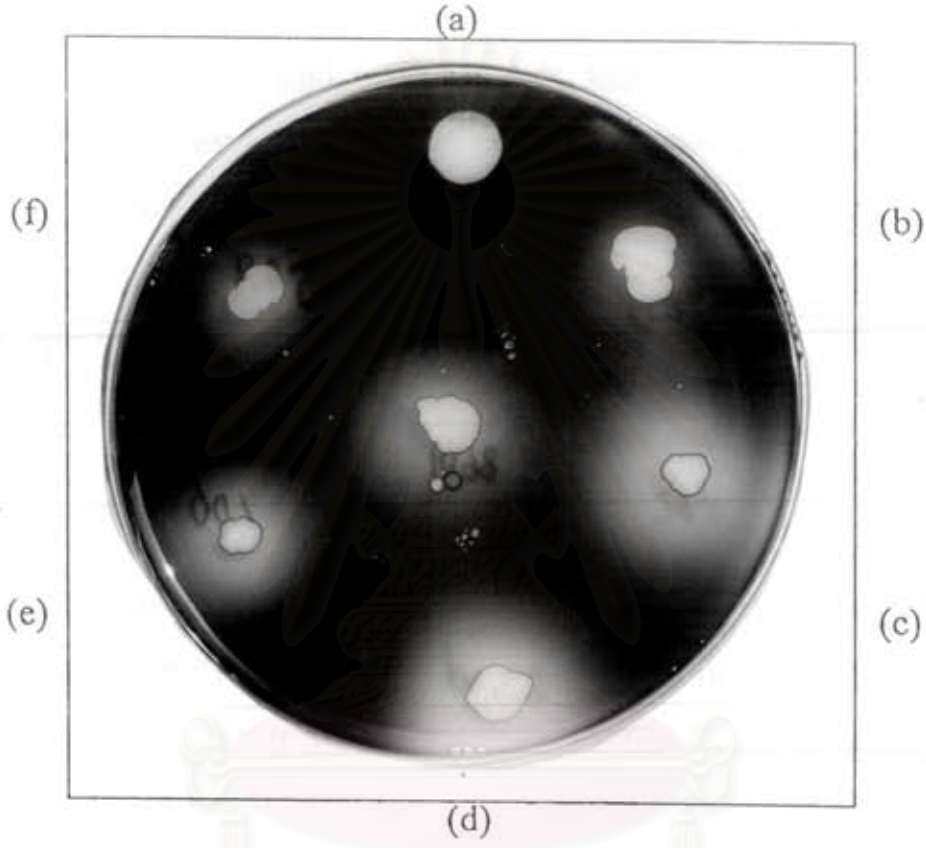


Figure 23 Example of transformants having amylolytic activity on LB-starch agar plate, pH 7.4, compared with DH5α containing pUC18
(a) DH5α containing pUC18, (b) 247, (c) 276, (d) 619, (e) 885, (f) 909, and middle well 1098

Dextrinizing activity assay

The selected transformants with amylolytic activity were then tested by dextrinizing activity assay. Cells were incubated in LB-starch medium at 37^0 C for 72 hours and centrifuged at 3000xg for 10 minutes. The supernatants were tested for dextrinizing activity. The results were shown in Table 13. 5 colonies (transformant numbers 14, 247, 276, 885, and 909) showed high dextrinizing activity but lower than that of *Bacillus* sp. A11 while *E. coli* DH5α containing pUC18 had no dextrinizing activity.

Phenol red inclusion complex test (PICT)

The transformants with high amylolytic and dextrinizing activities were tested with PICT which detected the production of CD from starch by the transformants, and it was more specific for CGTase. Cells were incubated in PM medium, pH 7.4, containing ampicillin, and incubated at 37 ^{0}C. The same 5 colonies as in dextrinizing activity assay could change color of PM medium. When compared to *Bacillus* sp. A11 which changed the color of PM medium from red to yellowish-orange, transformants 909 and 885 showed more orange color than 14, 247, and 276. This result demonstrated that each transformants had CGTase activity but less than *Bacillus* sp. A11. *E. coli*DH5α with or without pUC18 were used as control, and no color change from the original red were detected (Figure24).

Table 13 Dextrinizing activity of wild type and transformants

Cell	Dextrinizing activity (U/ml)
Bacillus sp.A11	45.00
DH5α	0.00
DH5α containing pUC18	0.00
13	0.05
14	3.17
15	0.03
22	0.37
247	1.93
276	1.48
279	0.56
282	0.41
376	0.88
495	0.75
529	0.11
561	0.72
619	0.00
648	0.29
729	0.47
735	0.09
804	0.54
885	2.14
909	11.02
1098	0.48

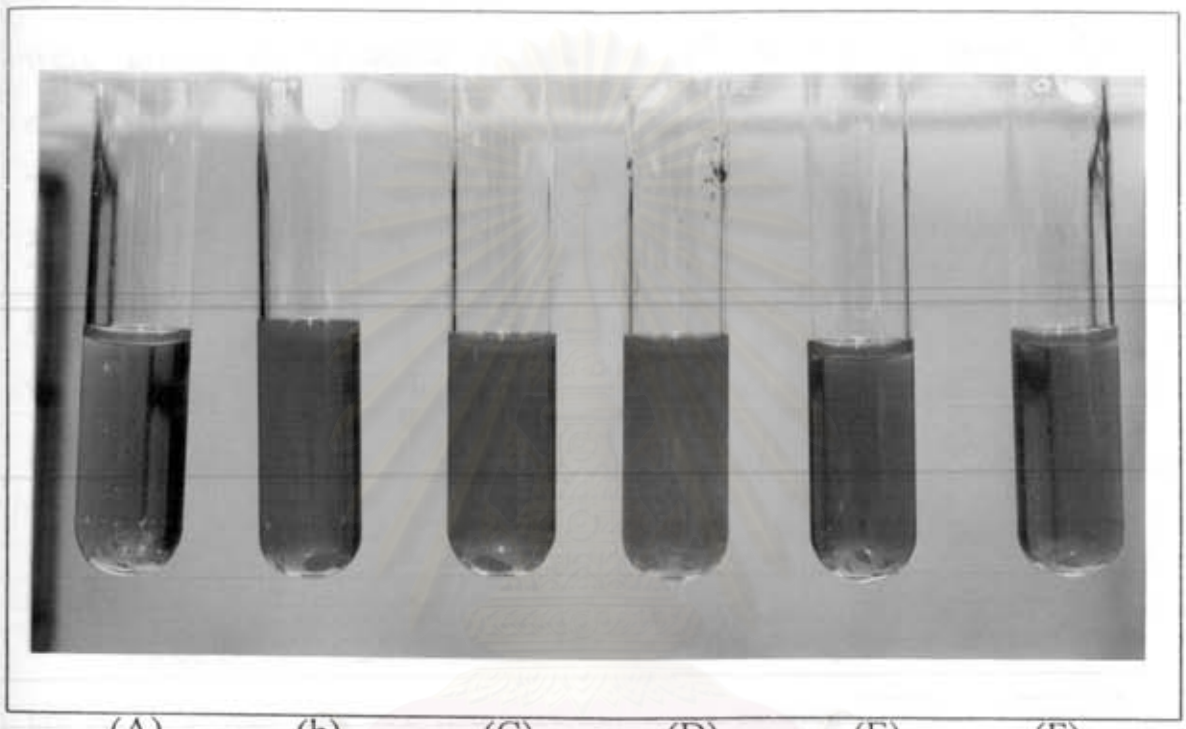


Figure 24 Detection of CGTase activity by PICT. Cells or transformants were incubated in PM medium at 37 ^{0}C for 72 hours. (A) *Bacillus* sp. A11, (B) DH5α, (C) DH5α containing pUC18, (D) transformant number 247, (E) 885, and (F)909.

CD-TCE assay

Five positive clones from the above assays were tesed for CD-TCE assay. The method precipitated CD products from CGTase action. Cells were grown in LB-starch medium at 37^0 C for 72 hours. After centrifugation at 3000xg for 10 minutes, supernatant were tested by CD-TCE assay. The result in Table 14 shows that transformants 909, 885, 14, 247, and 276 had the activity, expressed as 2^4, 2^1, 2^1, 2^0, and 2^0 dilution limit as compared to 2^6 of *Bacillus* sp. A11. The host *E. coli*DH5α with or without pUC18 did not give CD precipitation.

Detection of CGTase activity in recombinant colonies

Since *E. coli* transformants had CGTase activity lower than *Bacillus* sp. A11 , it was assumed that they might not secrete CGTase efficiently. To test this hypothesis, 909 cells were grown in LB-starch medium at 37^0 C for 72 hours. After centrifugation at 3000xg for 10 minutes, supernatant liquid S1 (extracellular enzyme) was collected. Cell pellet was incubated with 10 ml of lysozyme solution (25 mM Tris-HCl, pH8.0, 10 mM EDTA, and 2 mg/ml lyzozyme) at 0 ^{0}C for 2-3 hours. Supernatant liquid S2 (periplasmic space enzyme) was removed by centrifugation at 3,000xg for 10 minutes. The resuspended cells pellet (1 ml) was called S3. S1, S2, and S3 were tested for dextrinizing activity and the results were shown in Table 15. Table 15 shows that for *Bacillus* sp. A11, all CGTase activity was found to be extracellular. For transformant 909, 87.5% of activity was

Table 14 CD-TCE assay of wild type and transformant cells

Cell	CD-TCE (2^n)
Bacillus sp.A11	2^6
DH5α	none
DH5α containing pUC18	none
14	2^1
247	2^0
276	2^0
885	2^1
909	2^4

Table 15 Dextrinizing activity in various locations of cells

Cell	Total Dextrinizing activity (U)			Total activity (U)
	extracellular (100ml) (S1)	periplasmic (10 ml) (S2)	cell pellet (1 ml) (S3)	
Bacillus sp.A11	4500 (100%)	0 (0%)	0 (0%)	4500 (100%)
DH5α	0 (0%)	0 (0%)	0 (0%)	0 (0%)
DH5α containing pUC18	0 (0%)	0 (0%)	0 (0%)	0 (0%)
909	1102 (87.5%)	141.5 (11.2%)	16.20 (1.3%)	1259.7 (100%)

extracellular, while 11.2% was found in periplasmic space and 1.3% inside the cell. No activity was found in the *E. coli* with or without pUC18.

Analysis of CD by HPLC

The CD extracted from the reactions of CGTase enzyme from transformant 909 and *Bacillus* sp.A11 were analyzed by HPLC. The retention times of CD-products were compared to those of standard CDs. The retention times of standard α-, β-, γ-CD were 5.0, 5.97, and 7.2 minutes, respectively (Figure 25a). Those of oligosaccharides (G1-G7) were 2.88, 3.28, 3.88, 4.69, 5.68, 6.81, and 8.64 minutes, respectively (Figure 25b). The CD samples from *Bacillus* sp.A11, either with or without TCE extraction showed one major peak with retention time corresponded to that of standard β-CD (Figure 25c and d). CD obtained from transformant 909 with TCE extraction showed one major peak with a retention time identical to β-CD which was comfirmed by adding α-CD and β-CD as the internal standards (Figure 25e and Figure 26a). Figure 26b, the chromatogram of the reaction product from transformant 909 without TCE extraction, showed an overlap peak with retention time 5.08, 6.25, and 7.25 minutes, respectively. The retention time of the first and second peak matched those of α-and β-CD but third peak might be γ-CD or maltohexaose (G6) because standard γ-CD or G6 showed the same retention time value (Figure 26c, d, and e). The mixture of standard of α-, β-, γ-CD, and G6 were analyzed by HPLC, only three peaks were found (Figure 26f). This result showed that γ-CD and G6 could not be separated by this condition.

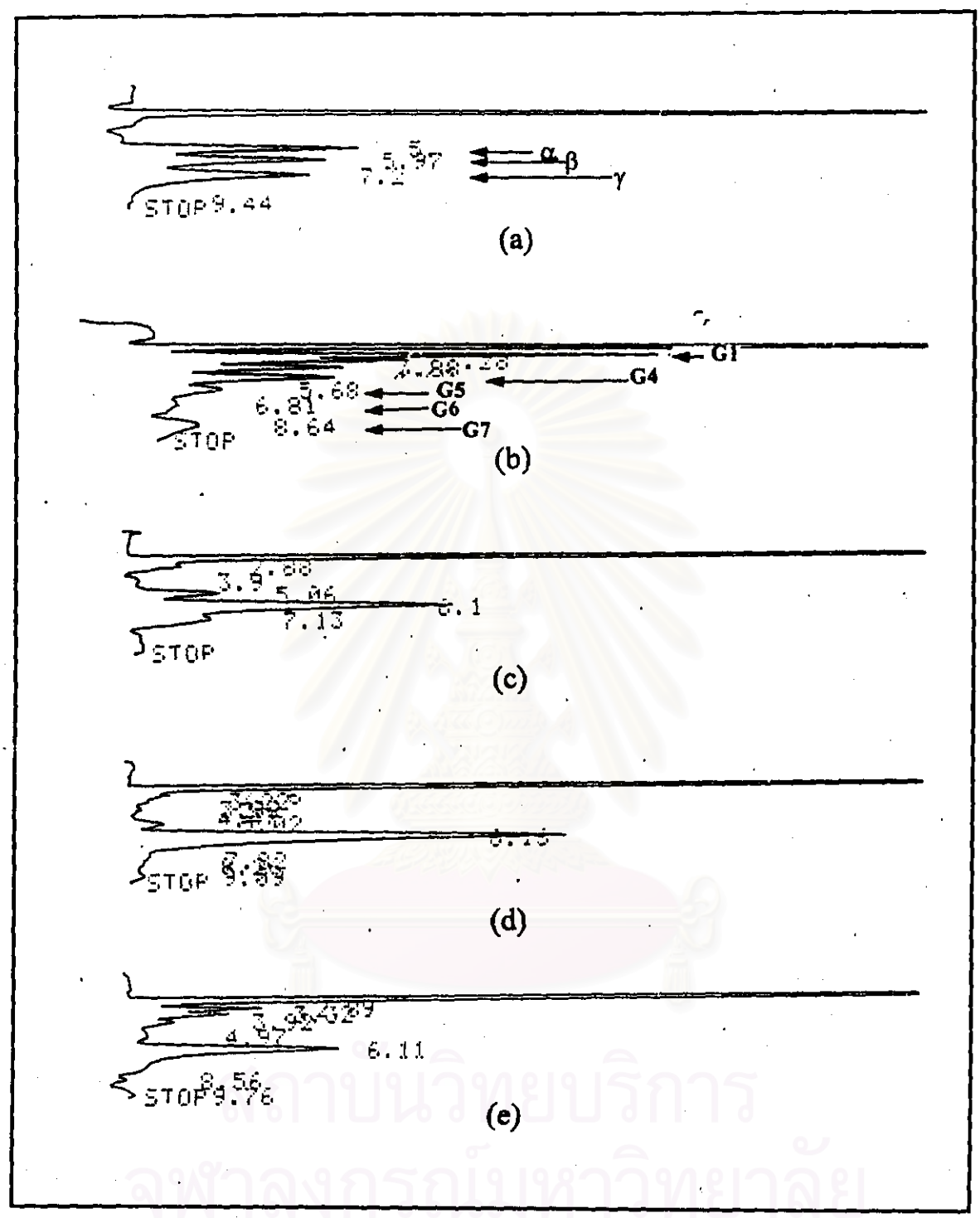


Figure 25 HPLC chromatograms of (a) Standard α-, β-, and γ-CD, (b) standard sugars (G1-G7), (c) reaction product formed from crude enzyme of *B.*sp. A11 without TCE extraction., (d) as in (c) with TCE extraction, (e) CD extracted from reaction of crude enzyme from transformant 909 with TCE. Supelco-NH_2 column was used. Acetonitrile: water (75:25) (v/v) was used as eluent at 2 ml/min flow rate.

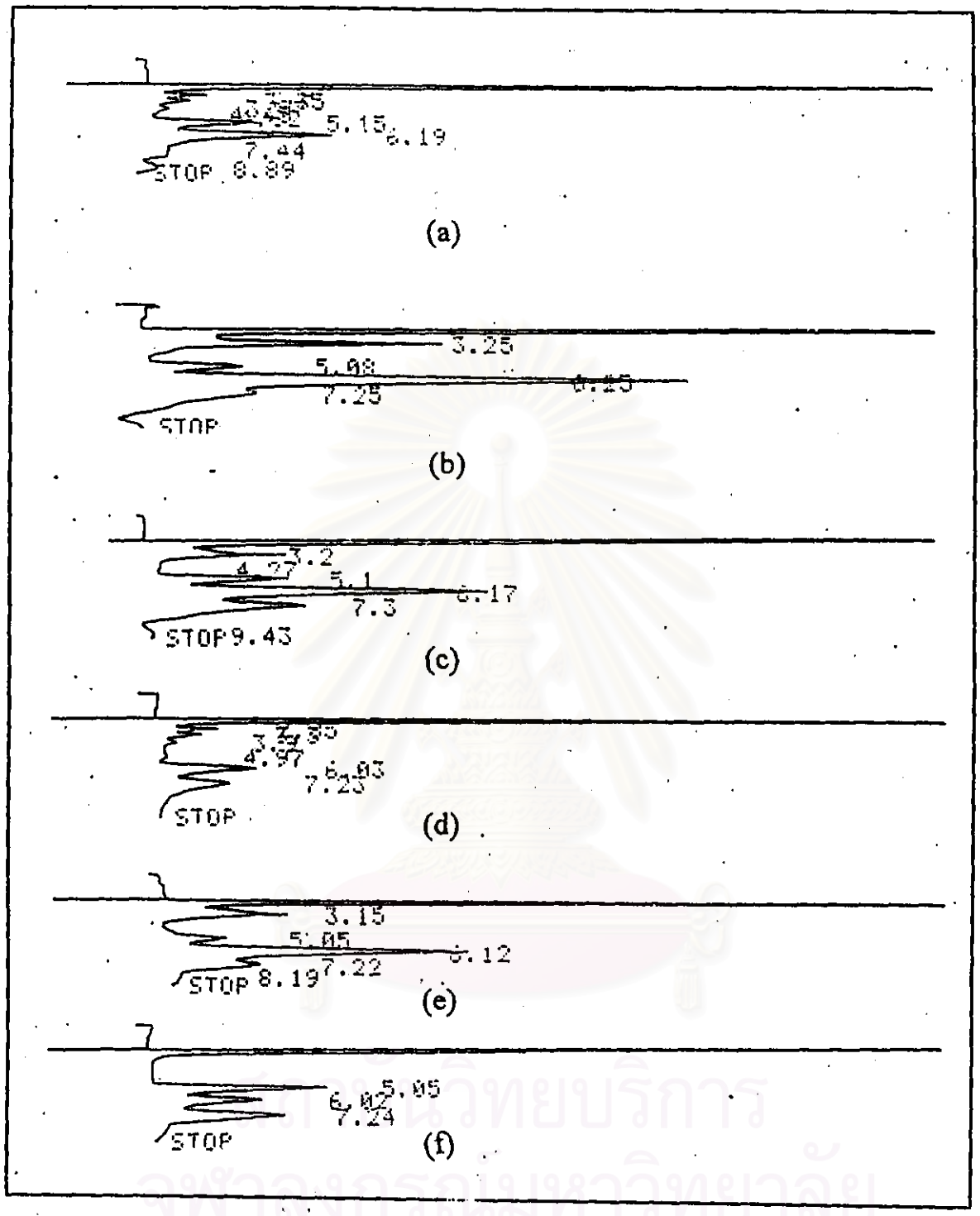
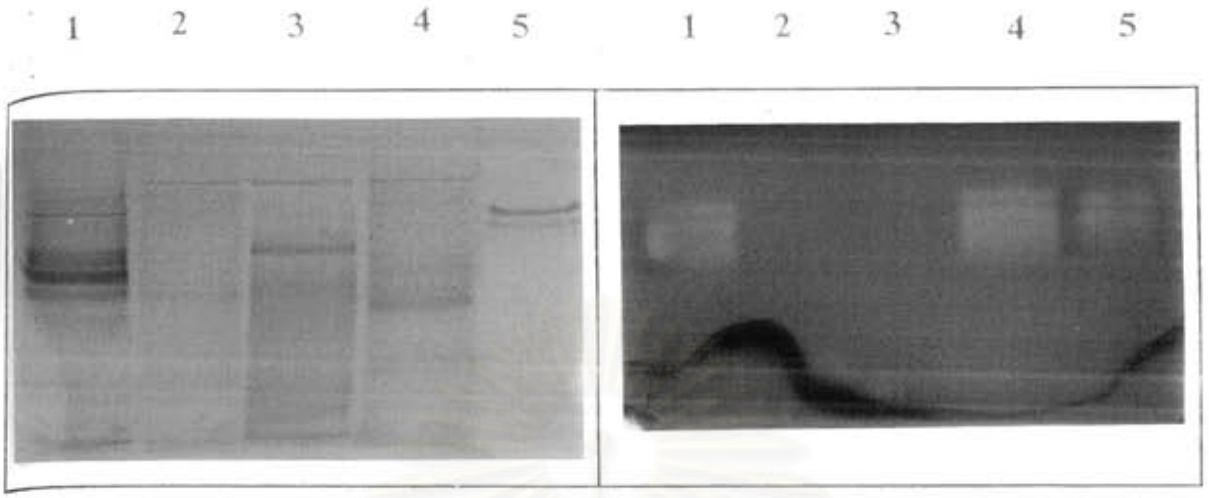


Figure 26 HPLC chromatograms of (a) reaction product formed from crude enzyme of transformant 909 with TCE extraction plus standard α-, β-, and γ-CD, (b) reaction product formed from crude enzyme of transformant 909 without TCE extraction., (c) as in (b) plus standard α-, β-, and γ-CD, (d) as in (b) plus standard γ-CD, (e) as in (b) plus standard G6 and β-CD, (f) standard α-, β-, and γ-CD plus G6. Supelco-NH_2 column was used. Acetonitrile: water (75:25) (v/v) was used as eluent at 2 ml/min flow rate.

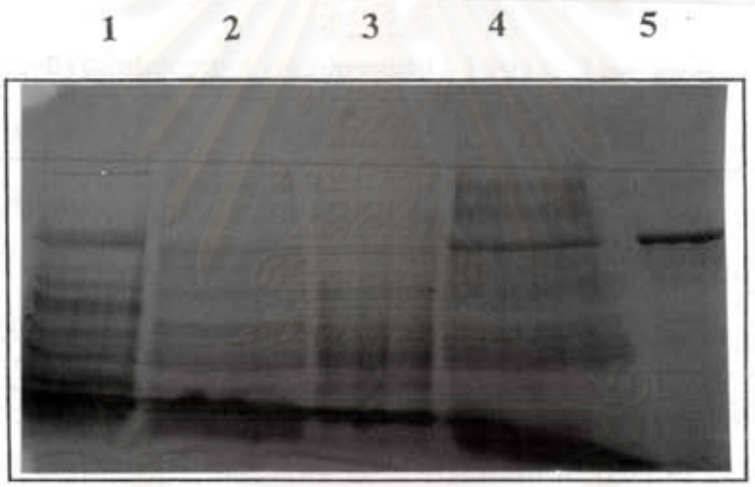
Analysis of CGTase enzyme from transformant 909 by native-PAGE and SDS-PAGE

DH5α contained pUC 18 and transformant 909 were cultivated in LB-starch containing ampicillin whereas *E. coli* DH5α and *Bacillus* sp. A11 were cultivated in LB-starch medium by incubating at 37^0 C for 72 hours. After centrifugation at 3000xg for 10 minutes, supernatant liquids were analyzed by non-denaturing-PAGE and SDS-PAGE, and compared with purified CGTase, as shown in Figure 27.

Protein staining was performed in Figure 27A. Each sample showed different protein patterns. The protein pattern of crude enzyme from transformant 909 may or may not correspond with crude from *Bacillus* sp. A11 and purified enzyme. Activity staining (amylolytic activity) showed that crude enzyme from *Bacillus* sp. A11 and transformant 909 exhibited bands in which one band was corresponded with purified CGTase (Figure 27B). SDS-PAGE showed an intense protein band in crude enzyme from transformant 909 which corresponded to CGTase from *Bacillus* sp. A11 and purified enzyme (Figure 27C). DH5α with or without pUC 18 could not produce this protein.



(A) (B)



(C)

Figure 27 PAGE of crude and purified CGTase from *Bacillus* sp.A11, crude enzyme from transformant (909), and crude extracts of DH5α and DH5α containing pUC18

(A) Coomasie blue protein staining [Lane 1: *Bacillus* sp.A11 (20μg), 2: DH5α(20μg), 3: DH5α containing pUC18(20μg), 4: transformant (909) (20μg), and 5: purified CGTase (2 μg)]

(B) Amylolytic activity staining by iodine solution. Lane 1-5, as in (A) (0.2 units of dextrinizing activity was loaded to each well)

(C) SDS-PAGE. Lane 1-5, as in (A)