CHAPTER IV

DISCUSSION

Purification of CGTase from *Bacillus* sp. A11

Bacillus sp. A11 was isolated from South-East Asian soil (Pongsawasdi and Yagisawa, 1987). It produces CGTase which converts starch to β-cyclodextrin (Techaiyakul, 1991). The enzyme was purified and characterized by our research group in the Department of Biochemistry.

The starch adsorption technique was widely selected as initial step in the purification of CGTase due to its specificity towards starch hydrolyzing enzyme, for example, CGTase from *B. macerans, B. circulans, B. ohbensis, and K. Pneumoniae* (Sin *et al., 1994*; Bender *et al., 1982*; Binder *et al., 1988*). CGTase was adsorbed after starch was added into crude enzyme and the adsorbed enzyme was eluted with buffer containing maltose. Ammonium sulfate precipitation and ion-exchange DEAE-cellulose column chromatography were usually performed to further purify enzyme.

In this work, an essentially pure enzyme preparation was obtained in a three-step purification procedure in which starch adsorption, ammonium sulfate precipitation, and DEAE-cellulose column chromatography using 0-0.3 M NaCl gradient were performed. At the DEAE-cellulose column chromatography step, elution peak containing CGTase activity was

separately pooled as 10 pools, which were named E1 to E10. Pool E1 to E10 were analyzed by non-denaturing and SDS-PAGE and specific activity was determined. When analyzed by non-denaturing PAGE, 2 major protein bands were found in pools E4 to E10 after protein staining while 2 activity bands were found in all pools (E1 to E10) when they were stained for starch hydrolytic and CD-forming activity. E5 to E8 showed higher specific activity than E1 to E4 and E9 to E10 and E5 showed the highest specific activity (Table 16). E5 to E8 were then pooled because all of these fractions showed 2 major protein bands corresponded to amylolytic and CD-forming activities when analyzed by non-denaturing PAGE and they were pools which possessed high specific activity. They also showed a single protein band on SDS-PAGE. E5-E10 pool was named DEAE-pool, yield of 30% was obtained with 44.8 fold of purity. The result showed that CGTase was purified in this experiment by three steps of purification while Techaiyakul (1991) used four steps: starch adsorption, ammonium sulfate precipitation, ion-exchange DEAE-cellulose column chromatography, and Sephadex G-100 column chromatography. She reported that enzyme was retarded and many percents of activity was lost during Sephadex G-100 column run. Rojtinnakorn (1994) used four steps of CGTase purification: starch adsorption, ammonium sulfate precipitation, ion-exchange DEAE-cellulose column chromatography, and Chromatofocusing column chromatography, and her result was similar to Techaiyakul's (1991). Kim (1996), linked anti-serum of CGTase, which was prepared by Rojtinnakorn (1994), to Sepharose 4B-chromatography matrix and used for enzyme purification. The purified enzyme from immunoaffinity column chromatography showed the same

Table 16 The specific activity of different pools from DEAE-cellulose column chromatography

Pool	Fraction number	Total activity* (U)	Total protein (mg)	Specific activity (U/mg)
E1	60-67	240	0.7	342.9
E2	69-71	800	1.1	727.3
E3	73-75	2,550	1.8	1,416.7
E4	76-79	7,400	3.8	1,947.4
E5	80	9,430	1.2	7,858.3
E6	82-84	23,400	3.5	6,685.7
E7	86-92	19,065	4.5	4,236.7
E8	93-98	7,380	2.2	3,354.5
E9	100-105	4,200	1.8	2,333.3
E10	107-120	3,300	2.8	1,178.6

* Dextrinizing activity

result as reported by Techaiyakul (1991) and Rojtinnakorn (1994).

Our purified CGTase showed 2 major protein bands with different intensity on non-denaturing PAGE, and both had been shown to have CGTase activity from the stainings for starch hydrolytic activity and CD-forming activity. Rojtinnakorn (1994) reported her CGTase obtained from DEAE-cellulose column exhibited 4 protein bands and found only 2 protein bands as CGTase after stained with starch hydrolytic activity and CD-forming activity. This may be because CGTase from this study was pooled at fractions which showed only 2 protein bands but Rojtinnakorn (1994) pooled all fractions with high activity. A protein band of M.W. about 72,000 daltons was found when detected with SDS-PAGE, similar to the result of Techaiyakul (1990), Rojtinnakorn (1994), and Kim (1996). The two protein bands on non-denaturing PAGE may be isozymes of 72,000 daltons or may be the result of different glycosylation state. This CGTase was proved to be a single polypeptide chain (Techaiyakul, 1991) which was similar to many CGTases such as those from *Bacillus* sp. No.38-2 and *Bacillus circulans* E192 (Kaneko *et al.*, 1988; Villete *et al.*, 1991). However, there has been some report that CGTases from *Bacillus macerans* IAM 1243 and *Bacillus megaterium* No.5 were dimeric proteins of similar subunit size of 145,000 and 66,000 daltons, respectively (Kobayashi et al., Kitahata and Okada, 1974). The CGTases from *Bacillus* strains (INMIA-T6, INMIA-T42, and INMIA-A71/19), *B. circulans* var. Alkalophilus, and *B. circulans* E192 showed 4-6, 2, and 6 subforms, respectively (Mattsson *et al.*, 1990; Bovetto *et al.*, 1992; Abelyan *et al.*, 1994).

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

Amino acid composition of CGTase from *Bacillus* sp.A11 and other microorganisms, was shown in Table 17. When the purified CGTase from *Bacillus* sp. A11 was determined for its amino acid composition, 40 mol% of the content was non-polar amino acids while 60 mol% was polar amino acids. Acidic amino acids: aspartic acid, asparagine, glutamic, and glutamine was rather high (25 mol%). Only 5 mol% was aromatic amino acids: phenylalanine, tryptophan, and tyrosine. It should be noted that aromatic amino acids of *Bacillus* sp. A11 CGTase was lower than those of other microorganisms. Sulpher-containing amino acids: cystein and methionine were low. CGTase of *Bacillus* sp. A11 was close to that of *Bacillus* sp. No.38-2 especially in the content of acidic and basic amino acids. Besides acidic and basic amino acids, other amino acids which were similar in all CGTases were Ser, Thr, Val, and Leu. When compared to other CGTases, our enzyme had significant difference in the amount of His, Pro, Tyr, and Met. His and Pro were higher wherease Tyr and Met were much lower (Bovetto *et al.*,1992).

Table 17 Amino acid composition of CGTases from *Bacillus* sp.A11 and other microorganisms

PERCENTAGE OF AMINO ACIDS (mol%)									
amino acid	*Bacillus* sp.A11	*Bacillus circulans* E192	*Bacillus macerans*	*Bacillus alkalo philus* 38-2	*Bacillus circulans* strain 8	*Bacillus* sp. Strain			
						INMIA A-T42	INMIA A-T6	INMIA A-A7/1	INMIA IA-1919
Asx*	15.42	16.62	14.70	16.06	15.64	17.10	14.80	14.90	16.00
Glx*	8.63	5.17	5.82	7.01	4.82	9.4	11.90	11.60	8.20
Ser	6.25	6.28	6.84	5.68	7.75	10.40	5.10	9.80	10.10
Gly	12.35	9.36	11.50	9.34	8.63	10.00	8.80	6.80	5.60
His	3.17	1.68	1.31	1.75	1.75	3.90	8.30	3.70	4.50
Thr	8.31	10.89	9.75	8.47	10.96	6.30	4.70	5.00	11.30
Arg	2.82	1.12	3.06	3.36	2.63	2.40	3.60	6.20	6.00
Ala	10.24	10.34	8.30	8.32	8.63	8.40	7.90	5.90	5.00
Pro	6.46	3.49	3.49	4.23	3.36	2.60	5.10	2.50	4.10
Tyr	1.55	3.91	4.95	4.96	4.68	2.60	5.40	6.00	6.00
Val	6.95	6.28	7.13	7.01	7.16	7.20	6.20	6.90	6.20
Met	0.44	1.26	1.75	2.04	1.61	1.80	2.50	1.40	1.50
Cys	0.29	0.00	0.00	0.29	0.29	0.20	0.00	0.00	0.00
Ile	4.28	5.17	5.24	6.28	4.82	4.80	3.60	3.80	3.70
Leu	5.54	5.59	5.83	5.26	5.41	5.90	5.50	6.60	3.70
Phe	3.56	5.59	4.66	4.68	5.41	3.60	2.20	4.50	5.70
Trp	0.16	2.65	1.89	1.90	2.05	n.d.	n.d.	n.d.	n.d.
Lys	3.56	4.60	3.78	3.36	4.39	3.90	4.40	4.40	2.40
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Reference	-	Villette *et al.*, 1991	Takeno *et al.*, 1986	Kaneko *et al.*, 1988	Bovetto *et al.*, 1992	Abelyan *et al.*, 1994			

Note n.d. = not determined

Asx* = aspartic acid plus asparagine

Glx* = glutamic acid plus glutamine

N-terminal amino acid sequence of CGTase from *Bacillus* sp. A11

N-terminal amino acid sequence of 20 amino acid residues of CGTase from *Bacillus* sp. A11 was determined by automated Edman degradation and compared with 8 strains of *Bacillus* sp., and *K. Pneumoniae* M5a1 as shown in Table 18. The amino acid sequence has been determined for N-terminal part of the active CGTase. The first five amino acids of *Bacillus* sp. A11, Ala-Pro-Asp-Thr-Ser, was similar to CGTases produced from *B.* sp. strain 1011, *B.* sp. strain 38-2, and *B.* sp. Strain 17-2 (Kaneko *et al.*, 1988: 1989; Kimura *et al.*, 1989).

When compared 20 amino acid sequence at the N-terminus of *Bacillus* sp. A11 to those of other bacterial strains, homologies with CGTases from *B.* sp. strain 1011, *B.* sp. strain 38-2, *B.* sp. strain 17-2, and *B. macerans*, and CGTases from *B. circulans* strain 8 and *B. licheniformis* were approximately 80 and 68 percent, respectively. No homologies with CGTase from *B. ohbensis* and little coherence (15%) with CGTases from *K. Pneumoniae* M5a1 and *B. stearothermophilus* were found (Bovetto *et al.*, 1992; Kaneko *et al.*, 1988: 1989; Kimura *et al.*, 1989; Takano *et al.*, 1986; Hill *et al.*, 1990; Sin *et al.*, 1991; Fujiwara *et al.*, 1992; Binder *et al.*, 1986). These values of homologies are closed to the values when *B. circulans* E192 sequence was compared to other strains: 80% homology with *B. alkalophilus* strain 38-2, 66% with *B. macerans*, and little coherence with *K. Pneumoniae* M5a1(Bovetto *et al.*, 1992). From these data, it can be concluded that the amino acid sequences of CGTases from *Bacillus* strains showed high homologies in the range of 50- 80% amino acid identity (Kaneko *et al.*, 1988).

Table 18 Comparison of 20 amino acid sequences at the N-terminal of CGTases of *Bacillus* sp. A11 and of other strains

Bacterial strains	1				5					10					15					20	Reference
Bacillus sp. A11	A	P	D	T	S	N	Y	N	K	Q	N	F	R	T	D	V	I	Y	Q	I	-
Bacillus sp. 38-2	A	P	D	T	S	V	S	N	K	Q	N	F	S	T	D	V	I	Y	Q	I	Kaneko *et al.*,1988
Bacillus sp. 1011	A	P	D	T	S	V	S	N	K	Q	N	F	S	T	D	V	I	Y	Q	I	Kimura *et al.*, 1989
Bacillus sp. 17-2	A	P	D	T	S	V	S	N	K	Q	N	F	S	T	D	V	I	Y	Q	I	Kaneko *et al.*, 1989
Bacillus macerans	S	P	D	T	S	V	S	N	K	Q	N	F	S	T	D	V	I	Y	Q	I	Takeno *et al.*, 1986
Bacillus circulans 8	D	P	D	T	A	V	T	N	K	Q	S	F	S	T	D	V	I	Y	Q	V	Bovetto *et al.*, 1992
Bacillus licheniformis	D	A	D	T	A	V	T	N	K	Q	N	F	S	T	D	V	I	Y	Q	V	Hill *et al.*, 1990
Bacillus ohbensis	D	V	T	N	K	V	N	Y	T	R	D	V	I	Y	Q	I	V	T	D	R	Sin *et al.*, 1991
Bacillus stearothermopholus	A	G	N	L	N	K	V	N	F	T	S	D	V	V	Y	Q	I	V	V	D	Fujiwara *et al.*, 1992
Klebsiela pneumoniae	A	E	P	E	E	T	Y	L	D	F	R	K	E	T	I	Y	F	L	F	L	Binder *et al.*, 1986

Cloning and expression of CGTase gene from *Bacillus* sp. A11 in *E. coli* DH5α

Our group has been able to clone CGTase in *E. coli* strain HB101, while cloning was not successful in *Bacillus* sp. (Siripornadulsilp, 1992; Vittayakitsirikul, 1994). A DNA fragment containing part of the CGTase structural gene from *Bacillus* sp. A11 was first cloned into pBR322 (low-copy number plasmid) and subcloned into pUC18 and pSE411, respectively. The inserted DNA fragment in pSE411 vector (pCSBC5) was 5.2 kb in size. It expressed in *E. coli* strain HB101 the CGTase activity, though lower than *Bacillus* sp. A11 (Siripornadulsilp, 1994). The 5.2 kb of inserted DNA fragment in pCSBC5 was bigger than CGTase gene (2-3 kb in size) from other bacterial strains. Since Techaiyakul (1990) reported that CGTase of *Bacillus* sp. A11 had MW. of 72,000 the structural gene should thus be around 2.0 kb in size (Wanna, 1995; Boonchai, 1995). Wanna (1995) studied the restriction maps of pCSBC8, and found that the same DNA inserts in pCSBC5 and pCSBC8 were in opposite direction. pCSBC16 was constructed by Boonchai (1995) it showed restriction map very similar to pCSBC5. *E. coli* with pCSBC16 had no CGTase activity. Wanna (1995) had tried to locate DNA region that contained of CGTase gene in pCSBC5. She found that 1.7 kb fragment from pCSBC5 expressed low dextrinizing activity. DNA sequencing of CGTase gene in pCSBC5 were determined. The DNA sequence showed no correlation with CGTase from other bacterial strains. Therefore, this study aims at construction of a new CGTase clone by using specific oligpnucleotide probes for the detection.

Chromosomal DNA extracted from *Bacillus* sp. A11 was partially digested with *Sau* 3AI because this restriction enzyme was widely used in the preparation of random DNA fragments for cloning (Siripornadulsilp, 1994; Takano *et al.*, 1986; Kato and Horikoshi, 1986; Kimura *et al.*, 1987; Kaneko *et al.*, 1988). 2-6 kb of DNA fragment was used in this study because CGTase genes from other bacterial strains were approximately 2-3 kb in size. Cohesive end of *Sau*3AI-digested DNA was compatible with cohesive end of *Bam*HI-digested pUC18. Therefore, the DNA inserts were cloned into the *Bam*HI site of pUC18. An *E. coli* strain DH5α was used as a cloning host. Selection of transformants harboring recombinant plasmids were performed by adding X-gal (substrate analogue of galactose) and IPTG in solid medium. The white colonies, due to the insertion, inactivation of lacZ′, were selected.

N-terminal amino acid sequence of CGTase from *Bacillus* sp. A11 compared with other bacterial strains showed high homology around 80% amino acid identity. Published C-terminal amino acid sequence of CGTases were also compared, and at least 60% amino acid identity was observed (Kaneko *et al.*, 1988:1989). The oligonucleotides were designed by decoding the N- terminal amino acid sequence to nucleotides. PNB 5′ HO-CAACAAGCA(G/C)AATTTCC-OH 3′ (17 bases MW. of 5027.4, melting temperature 44.0 ^{0}C) was designed. The 5' end nucleotide sequences and C-terminal amino acid sequences were directly obtained from published data. PCC 5′ HO-GTA(C/T)TATGATGTCAGCG-OH 3′ (17 bases MW. of 5247.9, melting temperature 44.0 ^{0}C) was designed from translation of C-terminus. Two oligonucleotides, each consisted of 17 nucleotides were synthesized. The two synthetic oligonucleotides were

labelled with [γ-^{32}P] by 5′ end labelling KIT (Pharmacia)and used as probes for screening of CGTase gene.

Probing of CGTase gene with oligonucleotides was performed and the gene was cloned in *B. ohbensis*. Three oligonucleotide probes, deduced from the N-terminal amino acid sequence of peptides from partial proteolytic cleavage, were used to identify the *B. ohbensis* CGTase gene (Sin *et al.*, 1991). Probing parts of CGTase gene from *Bacillus* sp. A11 was also reported, but the result was not clear. Wanna (1995) used inserted fragment from pCSBC8 as probe to hybridize with pCSBC5 by Southern blot hybridization. Boonchai (1995) used inserted fragment from pCSBC5 and pDS10 (2.3 kb, of α-CGTase gene in pUB110) as probe to hybridize with of pCSBC8 as well as selecting new recombinant DNA clone by Southern blot hybridization and Dot- blot hybridization.

The first screening of the recombinant DNA containing CGTase gene from *Bacillus* sp.A11 was perormed by colony hybridization. In this study, suppression of background hybridization was achieved by prehybridization the nylon membrane with a blocking reagent consisting of 2x Denhardt's reagent (0.5%(w/v) SDS and 100 µg/ml of yeast tRNA in place of denatured DNA). Hybridization signals with PNB and PCC were found in 483 and 405 colonies, respectively (data not shown). PNB showed stronger signal than PCC. This may be because PNB was directly deduced from the N-terminal amino acid sequence of *Bacillus* sp.A11 while PCC was from sequences comparison of CGTases from other bacterial strains. 360 positive clones showed signal with both PNB and PCC. Washing at 40 ^{0}C and 6xSSC gave rise to stronger specific signal than washing at room temperature (25 ^{0}C) and 6xSSC (Figure 21b and

22b) because higher temperature decreased background of [γ-^{32}P]-ATP and increased specific hybridization between probes (synthetic oligonucleotides) and template (recombinant plasmids). 360 transformant colonies were then transferred onto a new LB-starch agar plate containing 50 μg/ml of ampicillin (10 colonies per plate) and incubated at 37 ^{0}C, for 60 hours for the screening of clones possessing dextrinizing activity. 20 positive clones with clear zone around the colonies were detected after adding iodine solution.

The dextrinizing activity of these 20 colonies was quantitated. 5 out of 20 colonies exhibited dextrinizing activity higher than 1.0 U/ml (crude enzyme) and transformant 909 showed the highest activity but lower than *Bacillus* sp.A11 as shown in Table 13.

When compared the result from amylolytic activity on LB-starch agar plate (Figure 23) and dextrinizing activity from in-tube assay (Table 13), transformant 909 expressed smaller clear zone than other transformants but it gave the highest dextrinizing activity. As both assays are known to detect the same amylolytic activity of CGTase, the discrepency may be arised from different conditions of the techniques. Amylolytic activity on LB-starch agar plate was on solid medium that enzyme has to be excreted from the colony and penetrated into the agar. Difference of pH and temperature of both assays, amylolytic activity on LB-starch agar plate (pH 7.4, 37 ^{0}C) and dextrinizing activity (pH 6.0, 40 ^{0}C) might be the cause of discrepencies. Since optimum pH and temperature of CGTase of *Bacillus* sp.A11 were reported to be pH 6.0 and 40 ^{0}C (Techaiyakul, 1991), the result from dextrinizing activity assay should be more reliable.

PICT was also used to detect transformant cells containing CGTase. 2 transformants changes the color of PM medium from red to orange while *Bacillus* sp. A11 changed the medium color from red to yellow. The difference in color changing of PM medium between transformant cells and *Bacillus* sp. A11 may be due to different ratio of α-, β-, and γ-CD that converted from starch by CGTase. The α-, and β- CD could trap phenol red into CD-phenol red complex better than γ-CD (Park *et al.*, 1989). Techaiyakul(1990) reported that β- CD was the major product of CGTase from *Bacillus* sp. A11.

Ratio of α-, β-, and γ-CD of *Bacillus* sp. A11 was 1: 4.1: 1:1 and of transformant 909 was 1: 4.6: 1.6. Both cells showed β-CD higher than α-, and γ-CD but transformant 909 produced more γ-CD ratio than *Bacillus* sp. A11. Siripornadulsilp (1994) also reported that pCSBC5 expressed more γ-CD ratio than *Bacillus* sp. A11. CD-TCE assay which was more specific for CGTase than Dextrinizing activity and PICT, was performed in the last step of screening. Transformant 909 showed higher CD-TCE activity than other transformants but lower than *Bacillus* sp. A11. CD-TCE assay was more specific for β-CD than α-, and γ-CD. From all activities of CGTase measured, transformant 909 had lower activity only than *Bacillus* sp. A11. One possibility was that CGTase was low secreted from *E. coli*. Dextrinizing activity assay was used for localization of CGTase in transformant 909. 87.5%, 11.2%, and 1.3% of activity was found in extracellulur enzyme, periplasmic space, and cell pellet, respectively. No differences were detected when CD-TCE assay was used (data not shown).

CD-products of crude CGTase reaction mixture with and without TCE extraction were analyzed by HPLC. Those from *Bacillus* sp.A11 and transformant 909 showed one major peak, identified to be β-CD. Figure 26 showed that some products from reaction mixture of transformant 909 expressed were non-cyclic oligosaccharides (G1-G4) which is higher than those of *Bacillus* sp. A11.

The extracellular enzyme (crude enzyme) of *Bacillus* sp. A11, *E. coli* strain DH5α, *E. coli* strain DH5α containing pUC18, and transformant 909 were collected. Each sample was analyzed by non-denaturing PAGE and SDS-PAGE, compared with purified CGTase from *Bacillus* sp. A11. When non-denaturing PAGE was stained with protein staining, different protein patterns was found in each sample. After non-denaturing PAGE was stained with iodine staining, crude transformant 909 expressed clearly bands corresponded to purified CGTase from *Bacillus* sp. A11. Above results showed that transformant 909 produced CGTase. On the other hand, *E. coli* DH5α with or without pUC18 could not produce this protein.

From the above results, it was quite certain that transformant 909 contained CGTase gene. To determine the size and restriction map of the gene, further experiments were tried. The recombinant plasmid was isolated and analyzed by restriction enzyme digestion. As shown in Appendix M, recombinant plasmid could not be digested with *Eco*RI, *Bam*HI, *Kpn*I, *Sal*I, and *Hin*dIII that were found in multiple cloning site of pUC18. *Sca*I, *Pvu*II, and *Nde*I which were outside of the cloning site could digest the plasmid. Linear form of plasmid 909 showed shorter DNA fragment (about 2.4 kb in size) than pUC18 (2.7 kb in size).

The plasmid from transformant 909 could not be digested by many restriction enzyme tested, except *Pvu*II, *Nde*I, and *Sca*I. Siripornadulsilp (1994) had reported the CGTase recombinant plasmid which could not be digested with most enzymes except *Kpn*I and *Pst* I. The results from restriction analysis showed that the multiple cloning site of pUC18 may be deleted in some way. Most restriction endonuclease could not digest the plasmid 909, except *Pvu*II, *Nde*I, and *Sca*I which were outside of the multiple cloning site. The adjacent DNA around the multiple cloning site could be deleted. To test the hypothesis, plasmid 909 were analyzed by DNA sequencing. PNB, PCC, forward universal primer, and reverse universal primer were used as primer for DNA sequencing. The results in Appendix N showed that there were no sequencing ladder detected with plasmid 909. pUC18, as a sequencing control, gave sequencing signal when universal primer was used.

Only pUC18, as a sequencing control, indicated sequencing signal when universal primer was used. There were no sequencing signal with plasmid 909. The multiple cloning site might be deleted such that each primer could not anneal with template. However, retransformation of plasmid 909 into *E. coli* DH5α was performed to confirm that plasmid 909 contained inserted-DNA fragment of CGTase. The result showed that all transformant colonies expressed CGTase activity when detected by amylolytic activity on LB-strach agar plate and in-tube assays of dextrinizing and CD-TCE activities. This confirmed that plasmid 909 still had inserted-DNA fragment of CGTase gene.