CHAPTER IV

DISCUSSION

4.1 Purification of CGTase from *Bacillus* sp. A11

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

Purification of CGTase has been reported from *B. macerans*, *B. circulans*, *B. ohbensis*, and *K. pneumoniae* (Depinto and Campbell, 1968 ; Bender, *et al.*, 1982 ; and Binder, *et al.*, 1988). One common step in the purification procedures reported by the different laboratories was the use of starch adsorption, considering that the interaction between starch and the CGTase involves not only adsorption but also substrate-enzyme affinity type binding. In our research group, CGTase was adsorbed by starch added to the crude enzyme and the adsorbed enzyme was eluted with buffer containing maltose. Ammonium sulfate precipitation and chromatography on DEAE-cellulose (Laloknam, 1997) or immunoaffinity column (Kim, 1996) were usually performed to further purify the enzyme.

In this work, partial purified enzyme was obtained by corn starch adsorption technique. The result was similar to Malai (1994), in that the loss of CGTase at the adsorption step (activity remained in the supernatant after adsorption) was found to be approximately 7% while no activity was detected in the washing buffer. After CGTase

was eluted by maltose and concentrated by ultrafiltration, it was partial purified 120 folds with 78% yield and 145 folds with 23% yield, respectively. The high amount of CGTase lost in ultrafiltration step may cause by buffer shock from the rapid change of Tris-glycine buffer to distilled water.

4.2 Isolation of CGTase isozymes

In previous reports, CGTase isozymes were isolated by methods depended on their charge characteristics and specificity towards the ligand such as high-performance anion exchange chromatography (Makela, *et al.*,1988), isoelectric focusing (Mattsson, Meklin and Korpela, 1990), FPLC on a mono Q column (Bovetto, *et al.*, 1992) and affinity chromatography on a β-CD polymer (Abelyan, *et al.*, 1994). This work reported an alternative means of purification involving a preparative polyacrylamide gel electrophoresis in which the isozymes migrated differently in the gel depended on their sizes, shapes and charge characteristics. This technique involves separation of the protein bands on the polyacrylamide gel as usually performed on slab gel. After the separation on the gel, each protein band is eluted from the gel through the elution chamber and collected in test tubes. The advantage of this technique is that only partial purified enzyme is required for loading on the gel. Moreover, the elution chamber can separate these protein bands from each other, resulted in high purification of CGTase isozymes.

After elution of all protein bands from the gel, every other 5 fractions containing dextrinizing activity were analyzed again by non-denaturing PAGE, then the fractions which gave the same band, named as band 1, 2, 3, 4 and 5 were pooled. Through

this step, one CGTase isozyme was purified up to 211 folds, but the yield of all bands were very low (0.0004-1.8%). This may cause by the lost of activity during the running step on Prep cell or the low stability of the enzyme in the pH of elution buffer used.

Identification of CGTase isozymes

The pooled fractions were analyzed again by non-denaturing PAGE and activity stains were performed. The result showed that bands 1 to 4 exhibited both dextrinizing and CD-forming activity, while band 5 exhibited only dextrinizing activity. These results corresponded to the report by Rojtinnakorn (1994) in which pooled CGTase from ion-exchange DEAE-cellulose column chromatography showed 5 bands (1 to 5) with different intensity when stained for dextrinizing activity. CD-forming activity were detected only in bands 1 to 3 but not in bands 4 and 5. The purified enzyme from immunoaffinity column chromatography showed similar result (Kim, 1996). They speculated that bands 1, 2 and 3 were CGTase, whereas bands 4 and 5 may be other starch hydrolyzing enzymes or may be CGTase isozyme which had very low activity and cannot be detected under the detection limit of phenolphthalein. In those studies, the CGTase isozymes were co-purified and presence in the same preparation. Staining of the CGTase on the gel, therefore, showed only the bands with high CGTase activity. Increasing the amount of CGTase to increase the activity of bands 4 and 5 caused overloading of the gel and smearing in the phenolphthalein staining. In our study, each band of CGTase was separated from each other, so loading of 2.0 units of dextrinizing activity for each band yielded enough CD-products for the detection by phenolphthalein-methyl orange staining. The results showed that bands 1 to 4 can be detected by phenolphthalein while band 5 was detected only in

iodine stain. To ensure that band 5 lacked CD-forming activity, the amount of band 5 loaded on the gel for CD-forming activity stain was increases by 10 folds (based on dextrinizing activity). This experiment confirmed that band 5 did not contain CD-forming activity (Figure 10).

When bands 1 to 5 were analyzed by SDS-PAGE, a single protein band of molecular weight about 72,000 daltons were detected in all samples, similar to the purified CGTase of Techaiyakul (1991), Rojtinnakorn (1994), Kim (1996) and Laloknam (1997). Although they migrated differently on non-denaturing PAGE, they seemed to contain similar subunit with molecular weight of 72,000 daltons. Techaiyakul (1991) concluded that CGTase from *Bacillus* sp. A11 contained one polypeptide subunit. Other reports on *Bacillus* sp. NO. 38-2 and *Bacillus circulans* E 192 (Kaneko, *et al.*, 1988 ; Villette *et al.*, 1991) also showed similar results. However, there were reports that CGTases from *Bacillus macerans* IAM 1243 and *Bacillus megaterium* NO. 5 were dimeric proteins of identical subunit size of 66,000 and 145,000 daltons, respectively (Kitahata and Okada, 1974 ; Kobayashi, *et al.*, 1978). The CGTase from *B. circulans* E 192 showed 2 isoenzymes with different subunits of 33,500 and 48,500 daltons, respectively (Bovetto, *et al.*, 1992). CGTase isozymes (2-4 subforms) of *Bacillus* strains (INMIA-T6, INMIA-T42 and INMIA-A7/1) had molecular weights varied over the range from 25,000 to 50,000 daltons (Table 8) and it's interesting to note that the sum of the molecular weights of any two fractions obtained in each bacterial strain was equal to the reported molecular weight of the intact enzyme (Abelyan, *et al.*, 1994).

4.3 Some properties of the CGTase isozymes

Some properties of CGTase isozymes isolated from preparative gel electrophoresis were characterized. To determine whether all of these 5 protein bands appeared in polyacrylamide gel are glycoproteins, qualitative analysis by carbohydrate-specific periodic acid Schiff (PAS) staining method for polyacrylamide gel offers a good choice. In this stain, the periodic acid oxidizes the closed aldehyde groups in the polysaccharide conjugate, permitting the fuchsin sulfite to stain the polysaccharide the violet color. The result in this study showed that all of these 5 protein bands are glycoproteins. However, the staining did not represent the quantity of carbohydrate on the protein bands.

Having confirmed the presence of carbohydrate in each band, quantitative analysis by colorimetric test was also undertaken to measure their carbohydrate contents. The anthrone, dinitrosalicylic acid (DNS) and phenol-sulfuric acid methods are widely used. The former two methods had the disadvantage that, while they are satisfactory for free sugars and their glycosides, they are of limited use for methylated sugars and the pentoses. Moreover, the anthrone reagent is expensive and it is not stable in sulfuric acid solution (Morris, 1948 and Loewus, 1952). The phenol-sulfuric acid method which was used in this study is a direct total sugar assay. It can be used for the quantitative colorimetric microdetermination of sugars and their methyl derivatives, oligosaccharides and polysaccharides (Dubois, *et al.*, 1951). The method is simple, rapid, sensitive with permanent color produced, and gives reproducible results. By this method, the total carbohydrate contents for each band were shown to be different. This may be one of the reasons that each band of CGTase isozymes

migrated differently in native-PAGE while they migrated at the same position in SDS-PAGE.

The isoelectric points of these isozymes were determined by isoelectrofocusing gel in the ampholine pH range 3-7, comparing with standard pI markers (pI 2.5-6.5). The result showed that bands 1, 2, 3, 4 and 5 focused on the pI 4.73, 4.49, 4.40, 4.31 and 4.23, respectively. Charge differences smaller than one pH unit detected by IEF gel may arise from the carbohydrate content of glycoproteins or difference in a few amino acids in the polypeptide chain.

Rojtinnakorn (1994), using chromatofocusing column, reported the elution of CGTase in the pH range of 4.30–4.90 which were within the values obtained in this work. However, her results in native-PAGE showed that these 4 isozymes couldn't be successfully separated from each other, because bands 1 and 2, bands 2 and 3 and bands 3 and 4 were co-eluted, respectively.

The pI's of CGTase isozymes from other species have been reported. Makela and his group (1988) showed that the isoelectric points of CGTase fractions from an alkalophilic *Bacillus* strain ATCC 21783 were in the range between 4.55-4.90 as measured by ampholine electrofocusing and by chromatofocusing. CGTase of *Bacillus circulans* var. *alkalophilus* was studied by using isoelectric focusing (IEF) in immobilized pH gradient (IPG). The enzyme focused on IPG 4.5-5.4, was resolved into more than 6 subforms over the pH range 4.75-4.99. Fluorescence detection of CGTase activity in a strip of the focused gel was also undertaken (Mattsson, *et al.*, 1990). Bovetto and his group (1992) studied on *Bacillus circulans* E 192 and reported

on two isozymes separated by FPLC on a mono Q column, the products of each isozyme from various substrates analyzed by HPLC were mainly β-CD and their isoelectric points were estimated as 6.7 and 6.9. Therefore, majority of the reports on pI 's of CGTase isozymes were between 4.0-5.0.

The effect of pH and temperature on the isozyme activities were studied by both starch-dextrinizing activity (iodine method) and CD-forming activity (CD-TCE method). The former was widely used to measure the dextrinizing power of the enzyme which hydrolyzed α-1,4-glycosidic bond of starch substrate by detecting the decrease in color intensity of the iodine-starch complex. The latter, which is more specific for CGTase, detect the formation of cyclodextrin by observed CD-TCE precipitate between the upper starch solution layer and the lower TCE layer. The result showed that all isozymes studied in this work exhibited maximal dextrinizing activities in the range of pH 5.0-6.0 and 60°C whereas CD-forming actvities were maximum in the pH range of 6.0-7.0 and 40-50°C (Figures 14 and 15 a-b). This could possibly be explained by the inability of the iodine method to measure activity accurately when rapid cyclization of oligosaccharides occurs. The optimum pH and temperature of these isozymes were similar to the unfractionated (intact) enzyme reported by Techaiyakul (1991). Bands 1 and 2 seemed to be more stable in the pH range 6.0-7.0 where as bands 3 and 4 showed optimum activity only at pH 7.0. However, bands 3 and 4 (optimum temperature 50 and 60°C, respectively) seemed to be more heat stable than bands 1 and 2 (optimum temperature 40°C). Previous reports on optimum pH and temperature of partial purified CGTase (isozymes not separated) were pH 5.0-8.0 and 40-50°C.

The CGTase from *Bacillus* sp. A11 has been reported by our research group as β-CD producing enzyme (Techaiyakul, 1991 ; Malai, 1995 and Rattapat,1996). In this work, all of CGTase isozymes produced mainly β-cyclodextrin with the conversion percentage of 6.0 to 7.0% of the soluble starch, at a 2% (w/v) substrate concentration which coresponded to the previous study. The ratio of the reaction products (α:β:γ-CD) of each isozyme were slightly different. Bands 1 and 2 produced α-CD higher than γ-CD about 2 folds. Equal amount of α- and γ-CD were produced by band 3 while band 4 is the only isozyme which can produce more γ-CD than α-CD (Table 13). Further investigation of this preliminary results may lead to the possible selection of proper isozyme to produce specific type of CD products.

Malai (1995) reported that addition of β-amylase into the reaction mixture after CGTase reaction was stopped, help in removing the oligosaccharides which may interfere with CD's peak in HPLC. In this study, we investigated the experiment both with and without addition of β-amylase. The experiment omitting β-amylase was performed to analyze the kind of oligosaccharides actually produced by each isozyme. Bands 1 and 4 produced high amount of G7 which may lead to predominant disproportionation and cyclization reaction because the initial disproportionation of G6 and G7 yielded products that were cyclized at markedly higher rates. G1 and G2 which were high in bands 2 and 3 are commonly used as acceptor for the coupling reaction and must have some affinity for the acceptor binding-site of the enzyme. Furthermore, they served as acceptor for disproportionation of the substrate, but it is not clear why they did not inhibit the initial cyclization by interacting with the acceptor binding-site. This fact may be due to a rather low affinity for that site, or to an

alteration of the enzyme affinity by bound α-CD allowing the binding for the coupling reaction (Bender, 1985). For band 5, only linear oligosaccharides were detected with no CD-products. This confirms the result obtained from the native-PAGE that only amylolytic activity but no CD-forming activity appeared on the gel. It should be noted that the high peak with the retention time about 4.62-4.72 minutes which were not corresponded to any standard peak may be the peak of tris or glycine in the buffer or other oligosaccharide products such as cyclized G5. The possibility that this peak may be tris or glycine is less unlikely because it appeared as product of certain bands *e.g.* bands 2, 3, 4 only. Finding the retention time of tris or glycine in the same column can prove this. It is possible that this peak may be certain products which was produced by bands 2, 3 and 4 only. From the analysis of retention times of the standards CD's and linear oligosaccharide, this peak may be a product of cyclized G5.

Abelyan and his group (1994) reported the yields of CD-products obtained from several fractions of CGTase isolated from *Bacillus* strain INMIA-T42, INMIA-T6 and INMIA-A7/1. These fractions showed different product ratios which were also different from this report. Factors such as the kind of CGTase, the concentration of starch used and the technique employed for determining the percent formation of cyclodextrin may have contributed to the different values reported (Nakamura and Horikoshi, 1976). Several methods were employed for determining the amount of CD's. The indirect assaying of cyclodextrins is normally achieved by the glucoamylase method, with which the difference in total reducing sugar before and after the CGTase reaction is determined (Nakamura and Horikoshi, 1976). A more accurate assay was reported by Nakamura and Horikoshi (1976) involving the direct counting of ^{14}C-cyclodextrins

produced from ^{14}C-starch. The direct measurement of cyclodextrins by HPLC, as performed in this work, is another accurate means.

4.4 Amino acid composition of CGTase isozymes

Amino acid composition of CGTase isozymes from *Bacillus* sp. A11 and other strains were compared in Table 17. When the CGTase isozymes (bands 1-5) from *Bacillus* A11 were determined for their amino acid compositions, 40-50 mol% of the content were non-polar amino acids, while 50-60 mol% were polar amino acids. Acidic amino acids : aspartic acid and glutamic acid were rather high in bands 2-5 (22 mol%) while about 15 mol% was found in band 1. Only 7-10 mol% was aromatic amino acids : phenylalanine and tyrosine. Methionine, which is sulphur-containing amino acid was low. However, each band has some difference in their amino acid composition, such as threonine, which were rather high in bands 1, 2, 3, and 5 but was not found in band 4. On the other hand, histidine and proline which can be detected in small amount in bands 1, 2, 3 and 5 was rather high in band 4. The result in this study closely corresponded to the DEAE-cellulose purified enzyme reported by Laloknam (1997). When compared to CGTase isozymes from other sources, our isozymes had significant difference in the amount of Thr, Pro and Ala. These amino acids were higher in the isozymes of *Bacillus* sp. A11 than those of *Bacillus* strains INMIA-T42, INMIA-T6 and INMIA-A7/1. Phe and Arg were lower in the strains of INMIA-T6 and INMIA-T42, respectively.

Table 18. Comparative properties of CGTase isozymes from various strains

Properties	*Bacillus* sp. A11				*B. megaterium*		*B.circulans* *E192*		*B.* INMIA-T42				*B.* INMIA-T6		*B.* INMIA-A7/1	
	band1	band2	band3	band4	form1	form2	Fr.1	Fr.2	Fr.1	Fr.2	Fr.3	Fr.4	Fr.1	Fr.2	Fr.1	Fr.2
MW. (kD)*	72				75		78		35	31	28	25	38	30	50	44
pI	4.73	4.49	4.40	4.31	6.07	6.80	6.90	6.70	-	-	-	-	-	-	-	-
Opt. PH	6.0-7.0	6.0-7.0	7.0	6.0	5.2-6.2		5.5-5.8		6.5	7.5	6.5-7.0	6.5	6.5	7.0	7.0	6.0
Opt. Temp.	40°C	40°C	50°C	50-60°C	55°C		60°C		55°C				55°C		60°C	50°C
Product ratio (α:β:γ-CD)	10:18:5	9:18:5	5:18:5	5:18:7	-	-	-	-	2.9:3.5:1.0	5.2:3.1:1.0	4.8:3.5:1.0	5.2:3.4:1.0	4.8:3.8:1.0	5.4:3.7:1.0	1.0:27.0:4.0	1.0:58.4:7.4
Reference	-				Kitahata, *et al.* (1974)		Bovetto, *et al.* (1992)		Abelyan, *et al.* (1994)							

* The value determined by SDS-PAGE

Table 19. Amino acid composition of CGTase isozymes from various strains

Amino acid	Content (mol%)													
	Bacillus sp. A11						INMIA-T42[a]				INMIA-T6[a]		INMIA-A7/1[a]	
	P[b]	Band 1	Band 2	Band 3	Band 4	Band 5	F1	F2	F3	F4	F1	F2	F1	F2
Aspartate	14.4	10.0	13.3	13.2	12.2	14.6	19.4	18.7	12.9	17.3	12.5	17.1	14.1	15.7
Threonine	8.2	10.1	10.4	10.0	-	9.9	5.1	4.3	7.3	8.5	4.3	5.0	5.5	4.4
Serine	5.4	6.5	6.0	5.9	*	5.9	13.5	12.0	9.0	8.1	7.1	3.1	8.8	10.8
Glutamine	10.0	4.8	8.9	10.0	10.3	7.6	9.6	10.3	9.0	8.7	9.0	15.2	12.7	10.5
Proline	5.5	7.4	6.2	6.6	11.4	6.5	2.2	3.6	1.4	3.3	5.1	5.1	2.7	2.2
Glycine	8.9	*	*	*	*	*	11.6	14.1	9.0	9.5	4.8	12.7	4.4	9.2
Alanine	9.6	15.4	11.7	11.4	8.4	11.2	8.7	6.8	10.4	7.6	8.2	7.6	7.1	4.7
Cystine/2	Not determined						0	0.2	0	0.3	0	0	0	0
Valine	8.6	9.4	10.0	9.0	10.7	8.4	9.0	6.5	7.0	6.1	9.3	3.0	8.8	5.0
Methionine	1.1	2.2	1.4	1.1	1.3	1.4	0.3	0.4	1.8	0.7	3.3	1.7	1.4	1.5
Isoleucine	4.9	7.9	6.6	5.7	4.5	6.6	5.6	2.7	5.0	5.8	5.4	1.7	4.8	2.9
Leucine	6.3	6.5	6.2	6.5	8.3	6.6	6.7	4.7	7.9	4.4	8.0	3.0	8.8	4.4
Tyrosine	2.9	2.9	3.8	3.4	6.7	3.5	1.2	3.1	1.4	4.5	4.2	6.7	7.2	4.8
Phenylalanin	4.3	5.5	5.6	4.4	4.3	4.4	1.1	5.1	2.4	5.7	2.6	1.7	3.3	5.7
Lysine	5.6	4.5	4.8	5.3	5.4	4.3	1.6	1.2	6.8	3.8	4.7	4.0	4.2	4.6
Tryptophan	Not determined						Not determined							
Histidine	1.3	2.1	1.6	4.0	7.8	5.4	2.6	3.4	6.3	3.2	7.9	8.7	1.3	6.0
Arginine	2.9	4.8	3.5	3.8	8.6	3.9	1.8	2.9	2.4	2.5	3.6	3.7	4.9	7.6

[a] From Abelyan, *et al.* (1994)

P[b] Purified enzyme from Laloknam (1997) run in parallel

ND = Not determined

* Not calculated because of glycine contamination

From the results obtained from studies of the four isolated CGTase isozymes, it appeared that each of them migrated differently on non-denaturing polyacrylamide gel electrophoresis but appeared to have the same molecular weight on SDS-PAGE. The factors influencing movement of proteins in native-PAGE include shape, size, charge and glycosylation. We have proved that these isozymes were glycosylated at different degrees and contained some difference in their amino acid composition. These differences may contribute to the difference in charges of the four isozymes which resulted in different migration distance in the native gel and also the difference in pI's. Although their optimum pH's and temperatures for dextrinizing activity and CD-forming activity were only slightly different, they exhibited some difference in the amount of CD's and linear oligosaccharides produced. This may implicate certain difference in the preference of oligosaccharides in the disproportional reactions, which eventually resulted in some difference in their CD products. There is only one previous report by Abelyan, *et al.* (1994) of similar study on isozymes. They also found that each isozyme produced different CD's product ratio and their amino acid compositions differed in certain amino acids. To be able to conclude more on the difference of our isozymes especially in their CD's producing activities, further studies into the kinetics, utilization of different oligosaccharides as substrate, peptide mapping and N-terminal sequencing are suggested.