CHAPTER III

RESULTS

All data reported in this chapter, either in Table or graphic presentation, are averaged from at least 2-3 experiments performed at the same conditions.

Partial purification of CGTase from *Bacillus* sp. A 11

Crude CGTase was partially purified from the culture broth of *Bacillus* sp. A 11 by starch adsorption method as described in Section " Purification of CGTase" and Figure 5, Chapter II. By adsorbing of CGTase onto corn starch and washing with 10 mM Tris-HCl buffer containing 10 mM $CaCl_2$, pH 8.5, the loss of CGTase at the adsorption step (activity detected in Supernatant A) was found to be 6.8 % while no activity was detected in the washing buffer (Supernatant B). This indicated that 93 % of CGTase activity was adsorbed to corn starch in this condition. The adsorbed CGTase was then eluted from the starch cake with 0.2 M maltose in 10 mM Tris-HCl buffer containing 10 mM $CaCl_2$, pH 8.5, and dialyzed against 50 mM Sodium acetate buffer containing 5 mM $CaCl_2$, pH 6.0. Through these steps, the CGTase was partially purified to approximately 27-folds with 52 % yield and a specific activity of 2469 Units/mg protein, as shown in Table 7. The partial purified CGTase was then used for the production of cyclodextrins.

Table 7 Partial purification of CGTase from *Bacillus* sp. A 11.

Step	Volume (ml)	Enzyme activity		Total protein (mg)	Specific activity* (U/mg)	Recovery (%)	Purification fold **
		Dextrinizing (Total Units)	CD-TCE (dilution limit)				
Crude enzyme	1140	46,124	2^6	513	90	100	1
Partial purified enzyme	246	24,196	2^9	9.8	2,469	52	27

* Specific activity = $\frac{\text{Dextrinizing activity (units)}}{\text{Protein (mg)}}$

** Purification fold = $\frac{\text{Specific activity of each preparation}}{\text{Specific activity of crude enzyme}}$

Cyclodextrin production

1. Rice starch vs Soluble starch

Potato and corn starch were occasionally used in replacement of commercial soluble starch for cyclodextrin production in many laboratories. It would be of interest and economized to develop an effective process in cyclodextrin production from rice starch, Thailand ' s top agricultural product. Comparative preliminary investigation has been carried out on the use of rice and soluble potato starch for cyclodextrin production following the flowsheet in Figure 6. Both starches (20 %, w/v) were pretreated with 0.1 % (w/v) of α-amylase for one hour. After inactivating the enzyme, dissolved starches were incubated with the enzyme CGTase from *Bacillus* sp. A 11 (partially purified as described in Chapter II, 500 Units/g starch) for 17 hours. The reaction products were then analyzed by HPLC without further separation. Table 8 showed that rice starch yielded less total cyclodextrins than soluble starch which was mainly due to the difference in α-CD content. We later found that at high soluble starch content, significant amount of maltotetraose (linear G_4) occurred in the reaction products and could not be totally resolved from α-CD peak in our HPLC system. Product separation was studied and could be performed from Section 2-5, Chapter III as described in Section 4, Chapter II. At this point, β-CD was our main focus since CGTase from *Bacillus* sp. A 11 was reported to be β-CGTase (Techaiyakul, Pongsawasdi and Mongkolkul, 1992). We observed that the β-CD yields were relatively comparable (approximately 7 g%). Thus, the potential of using rice starch as the starting material for cyclodextrin production exists but optimization of production process should be

performed to increase cyclodextrin yield and to make rice starch worthwhile as the alternative for the expensive soluble starch.

2. Pretreatment of rice starch

When raw starch was used as the material for cyclodextrin production, pretreatment of starch with α-amylase was generally performed in order to obtain the proper substrate for CGTase. Pretreated and non-treated rice starch were then compared in this study as the starting material for cyclodextrin production. Table 9 showed that pretreatment of rice starch with α-amylase (0.1 % w/v, 1 hr) before incubation with CGTase (500 Units/g starch) rendered 100 times more total cyclodextrins yield than the non-treated rice starch. The result clearly indicated that the pretreatment step was necessary.

3. Optimization of pretreatment steps

3.1 Effect of α-amylase concentration and Dextrose Equivalent of starch on the formation of cyclodextrins

Rice starch (20 %, w/v) was pretreated with different amounts of α-amylase (0.05 to 1.0 %, w/v) for 1 hour and Dextrose Equivalent (DE) was determined as described in Chapter II. With α-amylase treatment, starch was hydrolyzed to DE values ranging from 3.3 to 16.5 (Table 10). Then the dissolved starch was incubated with CGTase (500 Units/g starch) for 17 hours and product separation was performed according to the flowsheet in Figure 6. As shown in Figure 8, the appropriate α-amylase concentration was 0.1 % w/v. Furthermore, Figure 9 revealed the effect of hydrolysis of starch on the production yield of cyclodextrins. It was found that total cyclodextrins increased when DE of dissolved

Table 8 Yield of cyclodextrins from rice and soluble potato starch.

Starch	Yield of cyclodextrins (g %)			
	α-CD	β-CD	γ-CD	Total CDs
Soluble starch (potato)	9.11	6.79	3.05	18.95
Rice starch	5.01	7.36	3.13	15.50

Table 9 Yield of cyclodextrins from pretreated and non-treated rice starch.

Treatment of rice starch	Yield of cyclodextrins (g %)			
	α-CD	β-CD	γ-CD	Total CDs
Pretreated	2.98	4.84	2.16	9.98
Non-treated	0.07	0.01	0.02	0.10

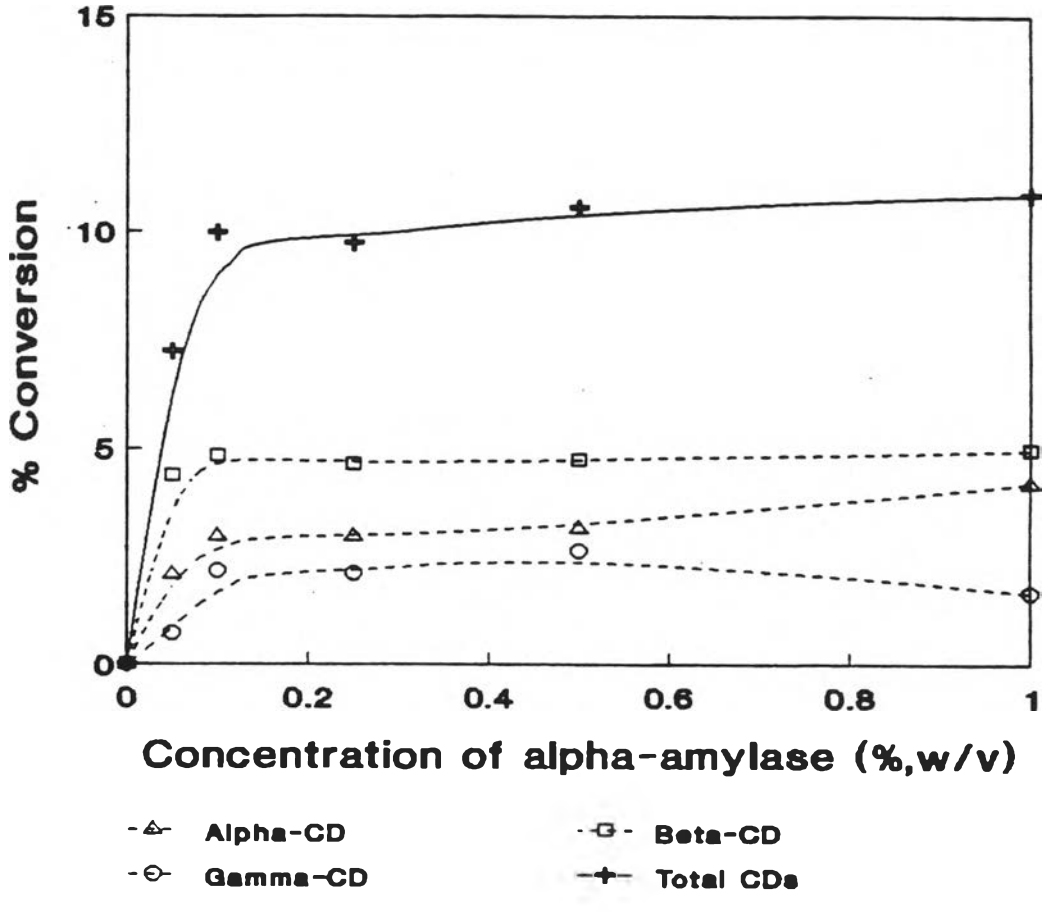


Figure 8 Effect of α-amylase concentration on the formation of cyclodextrins. Rice starch (20 %,w/v) was pretreated with α-amylase for 1 hour, then the dissolved starch was incubated with CGTase (500 Units/g starch) for 17 hours.

Table 10 Dextrose Equivalent values of treated starch. (Rice starch was pretreated with bacterial α-amylase for an hour).

% w/v α-Amylase treated	DE of treated starch
0.05	3.3
0.1	6.3
0.25	8.4
0.5	9.8
1.0	16.5

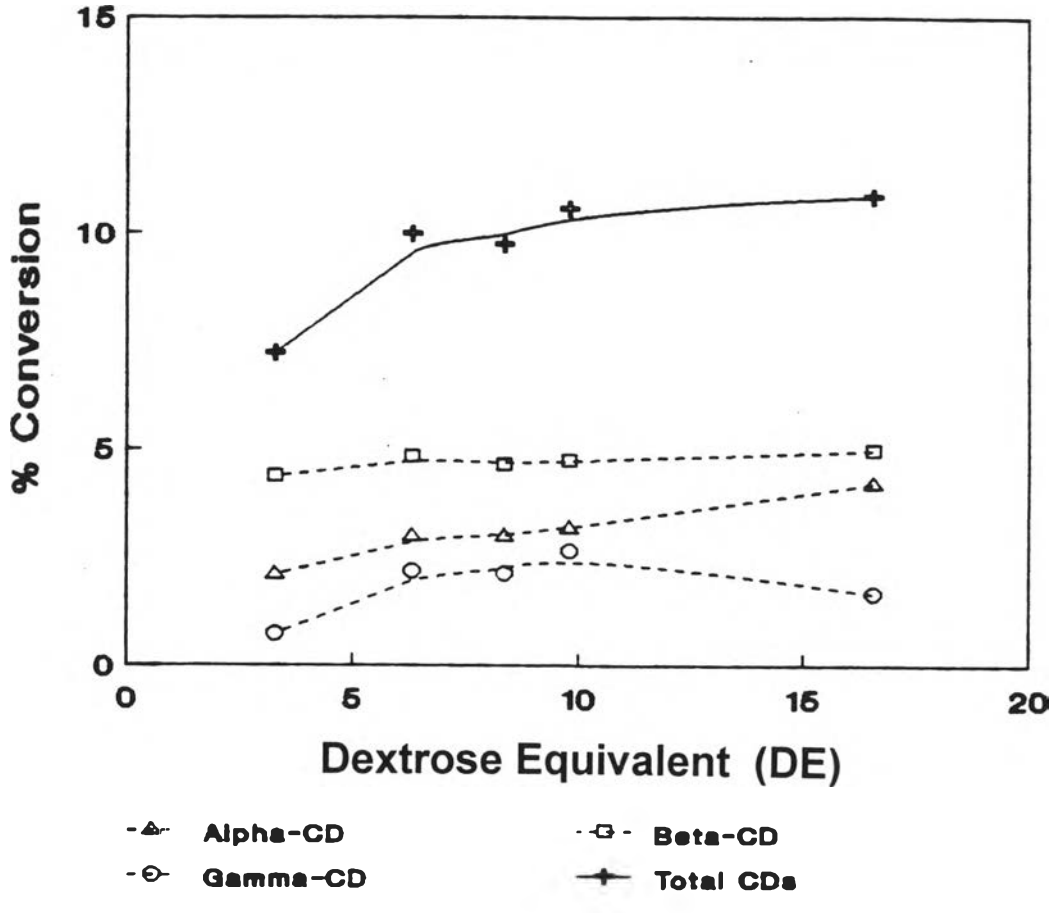


Figure 9 Relationship between Dextrose Equivalent (DE) of starch and the formation of cyclodextrins. Conditions were as described in Figure 8.

starch increased, especially at DE< 10. The yield of α-CD increased with increasing DE while β-CD yield remained stable and γ-CD yield tended to decrease when DE was more than 10. Thus, considering the total amount of cyclodextrins, the high ratio of β-CD produced and the minimum concentration of α-amylase required, 0.1 % by weight of the enzyme was chosen as the most suitable concentration for the pretreatment step.

3.2 Effect of incubation time of pretreatment of starch on the formation of cyclodextrins

After the optimum concentration of α-amylase was chosen at 0.1 % (w/v), the pretreatment time was varied from 15 to 60 minutes and the Dextrose Equivalent (DE) was determined as described in Chapter II. The dissolved starches obtained had DE values of 6.16 to 7.21. Thus, varying the concentration of α-amylase had more effect on the DE values of starch than varying the pretreatment time. Then the dissolved starch was incubated with CGTase and product separation was performed as in 3.1. As shown in Figure 10, total cyclodextrins and each cyclodextrin yields increased in the first 15 minutes, then leveled off. From the result, the appropriate incubation time of pretreatment of starch was at 15 minutes.

3.3 Effect of starch concentration on the formation of cyclodextrins

The effect of starch concentrations on the production yield was investigated. In this work, different amount of rice starch, 1.5, 2.0, 2.5, 5.0, 10, 20 and 30 % (w/v), were used for cyclodextrin production, using the optimum pretreatment condition of 0.1 % (w/v) of α-amylase for 15 minutes. Then, the dissolved starch was incubated with CGTase and product separation was performed

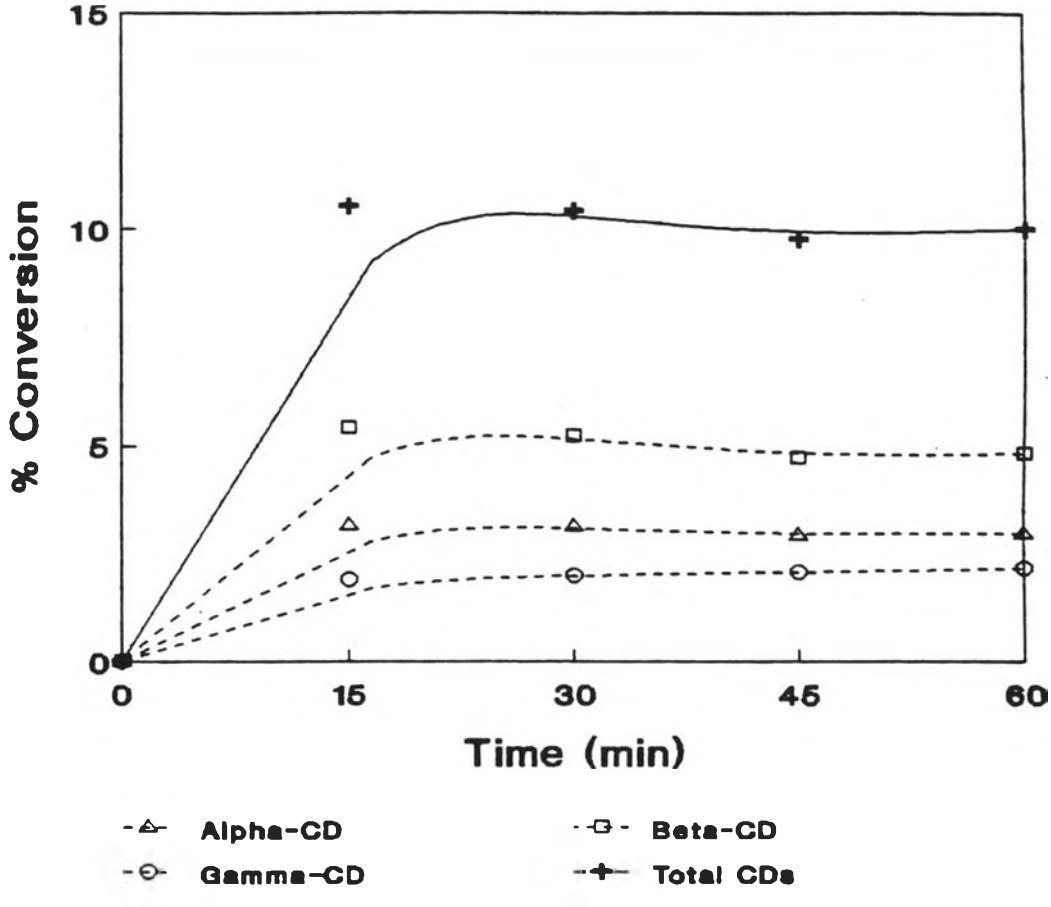


Figure 10 Effect of incubation time of pretreatment of starch with α-amylase (0.1 %, w/v) on the formation of cyclodextrins. Conditions were as described in Figure 8.

as in 3.1. As shown in Figure 11, the total amount of cyclodextrins produced increased from 10 to 26 g/l with increasing concentrations of rice starch from 1.5 to 30 % w/v, while % conversion or production yield decreased from 60 to 7 percents. It should also be noticed that the product ratio of α: β: γ differed when starch concentration was varied (Table 11). At low concentrations of rice starch (from 1.5-5.0 %), α-CD was the main product; while at high concentrations (from 10-30 %), β-CD was the major cyclodextrin formed. From this experiment, the optimum concentration of starch was selected to be 10 % (w/v) in consideration of the production yield as % conversion, total amount of cyclodextrins and the major form of cyclodextrin produced.

4. Optimization of production steps (without complexing agent)

4.1 Effect of CGTase concentration on the formation of cyclodextrins

In order to determine the optimum ratio of enzyme to starch, CGTase was varied and the production of cyclodextrins was investigated following the protocol in Figure 6. The concentration of rice starch was fixed at 10 % (w/v) and the pretreatment conditions were as optimized : 0.1% (w/v) of α-amylase for 15 minutes. Treated rice starch was incubated with varying amounts of partially purified CGTase (15, 25, 50, 125, 250, 500, 1000 Units/g starch) for 17 hours. Product separation was also performed. Figure 12 showed that only 25 Units CGTase/g starch was enough for maximum cyclodextrin production. At low amount of CGTase (< 200 Units/g starch) β-CD was the major product, while at high amount of CGTase (200-1000 Units/g starch) the increase of α-CD was observed. CGTase at 25 Units/g starch was chosen for cyclodextrin production in consideration of total cyclodextrins and β-CD obtained.

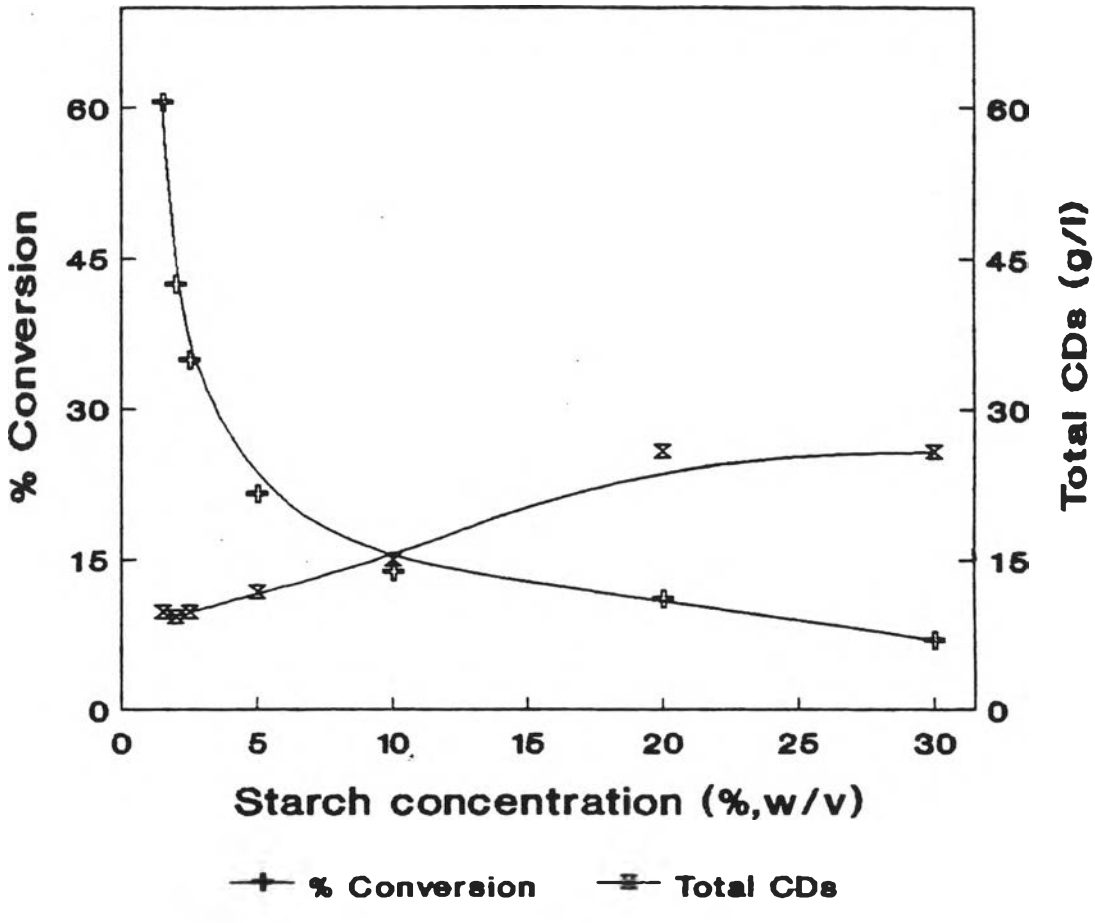


Figure 11 Effect of starch concentration on the formation of cyclodextrins. Conditions were as described in Figure 10 except that 15 minutes was used for the pretreatment time.

Table 11 Effect of starch concentrations on cyclodextrin production.

% Starch	Yield of cyclodextrins				Total CDs
	% Conversion				
	α-CD	β-CD	γ-CD	Total CDs	
1.5	33.64	21.38	5.49	60.52	9.65
2.0	21.73	16.27	4.40	42.40	9.22
2.5	18.37	14.06	2.43	34.86	9.67
5.0	11.72	7.89	1.94	21.55	11.70
10	6.18	5.97	1.61	13.76	14.96
20	3.55	5.27	2.45	11.07	25.74
30	2.41	3.20	1.35	6.96	25.78

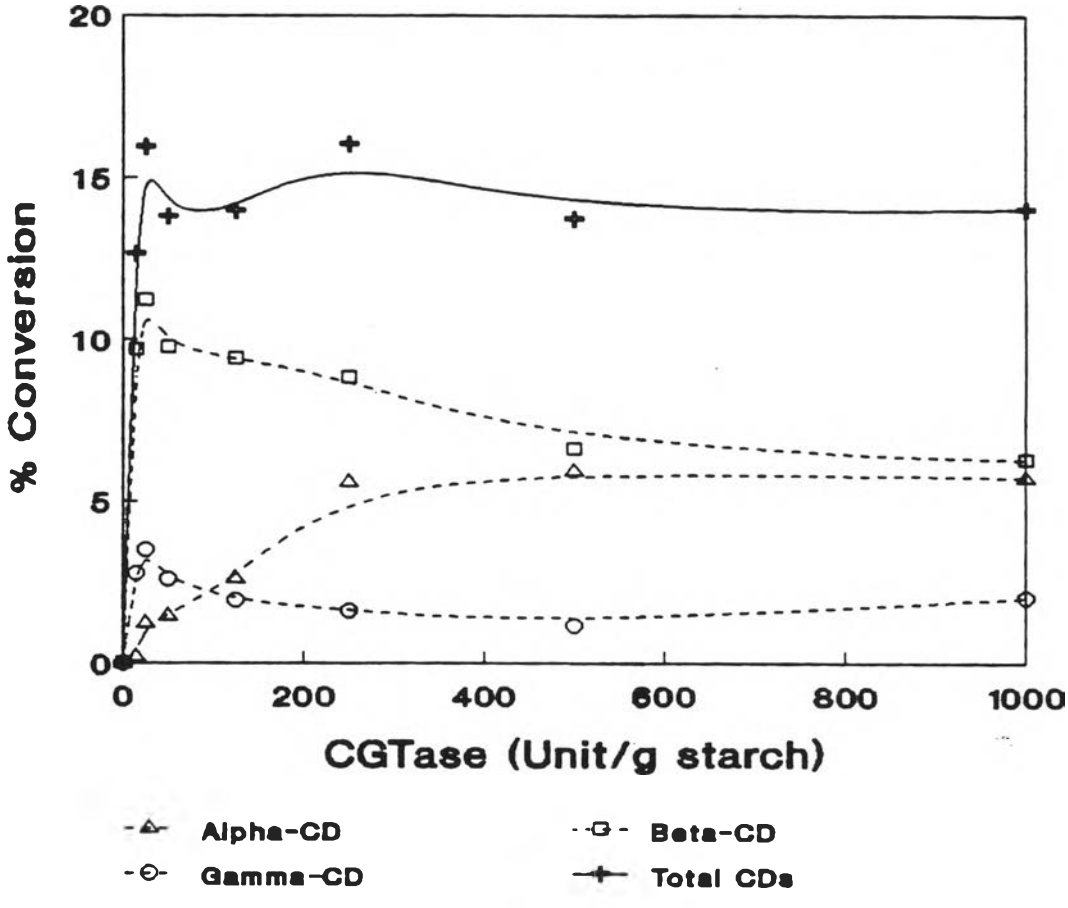


Figure 12 Effect of CGTase concentration on the formation of cyclodextrins. Conditions were as described in Figure 11 except that 10 % (w/v) of rice starch was used as the starting material.

4.2 Time course of cyclodextrin production

Time course of cyclodextrin production was also determined. Pretreated rice starch at the optimized conditions was incubated with 25 Units/g starch of CGTase. The production time was varied from 3 to 24 hours. Figure 13 showed that the production yield rapidly increased in the first 9 hours, then leveled off, while β-CD yield was maximum at around 12 hours.

When cyclodextrin production was performed according to protocol in Figure 6 at the optimized conditions of the " Pretreatment " and " Production " steps chosen from Section 3 and 4, the total cyclodextrin yield was 19 g%, with β-CD as the major product, constituted for 15.6 g%.

5. Optimization of production steps (with complexing agent)

5.1 Effect of complexing agent on activity of CGTase

Production of cyclodextrins in the presence of complexing agent was performed. In this aspect, it is of importance to first check the effect of each complexing agent on the activity of CGTase. Toluene, cyclohexane, trichloroethylene (TCE), methyl-ethyl ketone (MEK), naphthalein (Nap), n-decyl alcohol (decanol), or ethanol at 5 % (v/v) was incubated with CGTase at 50°C for 24 hours. Dextrinizing assay was determined as described in Chapter II from initial time to 24 hours. As shown in Figure 14, the activity of CGTase was not affected by all complexing agents tested except for n-decyl alcohol. The activity in the presence of n-decyl alcohol decreased about 30 % at 24 hr of incubation.

The result from this experiment suggests that all chemicals tried except n-decyl alcohol could be used as the complexing agent in the process of cyclodextrin production. Selection of the best complexing agent and optimization of

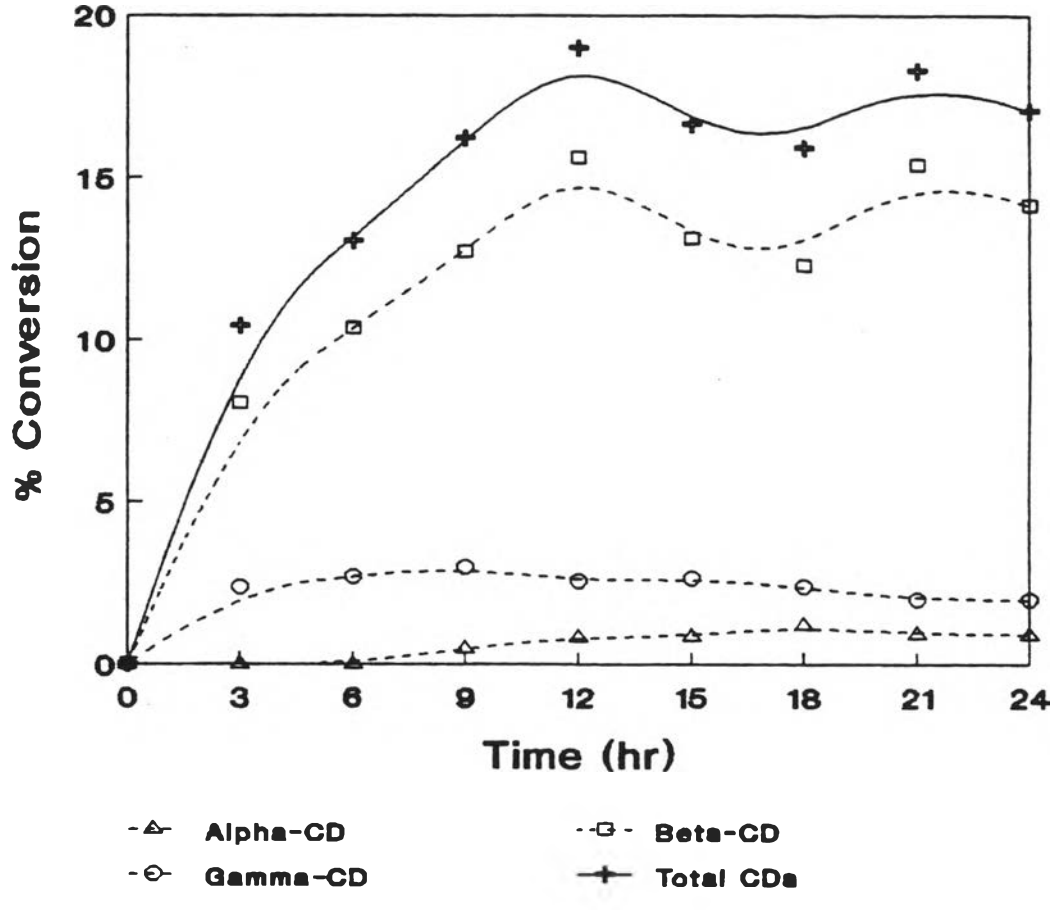


Figure 13 Time course of cyclodextrin production without complexing agent. Conditions were as described in Figure 12 except that the dissolved starch was incubated with 25 Units CGTase/g starch.

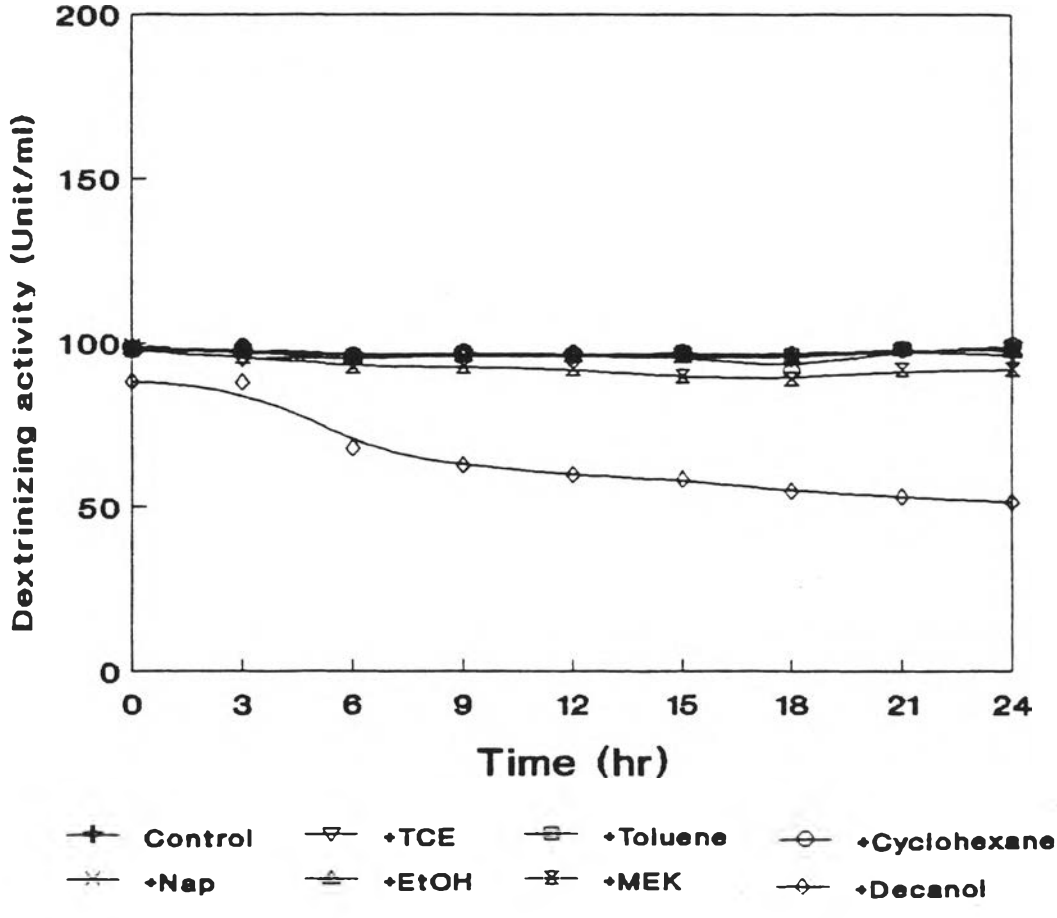


Figure 14 Effect of complexing agents on CGTase activity. Each complexing agent at 5 % (v/v) was incubated with CGTase at 50°C for 24 hours. Dextrinizing assay was performed at time intervals.

production steps was then performed.

5.2 Effect of CGTase concentration on the formation of cyclodextrins in the presence of complexing agent

Each complexing agent described in 5.1 at 5 % (v/v) was added after 30 minutes of incubation of treated starch (obtained from the optimized pretreatment conditions) with the CGTase enzyme which was varied from 25-1000 Units/g starch. Then the reaction mixture was incubated until 17 hours and product separation was also performed. Table 12 showed that low amount of CGTase (25-50 Units/g starch) was better in cyclodextrin production than high amount (500-1000 Units/g starch). Moreover, it was found that at low amount of CGTase, β-CD yield was maximum while at high amount of CGTase, the increase of α-CD was observed, the result of which was the same as the production without complexing agent (4.1). Cyclohexane, trichloroethylene, and toluene were shown to be very good complexing agents since they increased production yield from approximately 16 g% to 34 g%. However, cyclohexane gave higher yield at lower CGTase activity when compared to trichloroethylene and toluene.

All complexing agents described yielded β-CD as the major product. The respective order of the complexing agents which produce high to low yield of β-CD are : cyclohexane > trichloroethylene > toluene > naphthalein (Nap) > n-decyl alcohol > ethanol > methyl-ethyl ketone (MEK) > cyclohexane-MEK > Nap-MEK. In this experiment, binary complex and ternary complex formation between CD-complexing agent (such as CD-cyclohexane) and complexing agent 1- CD - complexing agent 2 (such as cyclohexane-CD-MEK) was compared. The result showed that all binary complex formation gave higher cyclodextrin yield than the

Table 12 Production yield of cyclodextrins in the presence of complexing agent at different concentrations of CGTase.

Complexing agent	Yield of cyclodextrins (g %)															
	CGTase (Units/g starch)															
	25				50				500				1000			
	α	β	γ	T	α	β	γ	T	α	β	γ	T	α	β	γ	T
None*	1.23	11.20	3.51	15.94	1.47	9.74	2.59	13.80	5.94	6.61	1.17	13.72	5.73	6.28	2.02	14.03
Cyclohexane	0.32	33.83	-	34.15	0.52	31.57	-	32.09	3.40	25.55	-	28.95	2.53	20.61	-	23.14
Trichloroethylene	0.56	30.76	-	31.31	0.72	33.58	-	34.30	5.39	29.47	-	34.86	3.76	19.46	-	23.22
Toluene	0.56	26.24	-	26.80	0.58	31.38	-	31.96	5.14	27.71	-	32.85	3.72	19.97	-	23.69
Naphthalein	0.40	26.50	-	26.90					3.79	18.01	-	21.80				
n-Decyl alcohol **	0.36	24.62	-	24.98					9.11	18.14	0.94	28.19				
Ethanol	-	16.66	-	16.66												
Methyl-ethyl ketone	-	3.86	-	3.86												
Cyclohexane-MEK	-	2.64	-	2.64												
NAP-MEK	-	1.55	-	1.55												

α = α-CD, β = β-CD, γ = γ-CD, T= Total CDs

* The result of cyclodextrin production with no complexing agent was from 4.1.

** This complexing agent inhibited dextrinizing activity of CGTase by 30 %.

ternary complex formation system tried.

From the data obtained, CGTase (25 Units/g starch) and the complexing agent ; cyclohexane, were chosen on consideration of total cyclodextrins and β-CD yield.

5.3 Time course of cyclodextrin production in the presence of complexing agent

Time course of cyclodextrin production in the presence of complexing agent (cyclohexane) was studied. The starch concentration, the pretreatment conditions, and the CGTase were as optimized. The production time was varied from 3 to 24 hours, while cyclohexane (5 % v/v) was added to the reaction mixture after 30 minutes of incubation. Figure 15 showed that there was an increase in the initial production rate of cyclodextrins, and the total amount of cyclodextrins reached a maximum peak at 18 hours. It should be noticed here that the amount of α- and γ-CDs were relatively low. Nearly all of the cyclodextrins were β-CD.

Figure 16, comparing Figure 15 to Figure 13, showed that the maximum amount of total cyclodextrins and β-CD (at 18 hours) in the presence of cyclohexane was about twice of that without complexing agent. Production of β-CD was especially increased in the presence of cyclohexane.

5.4 Effect of complexing agent concentration on the production yield of cyclodextrins

Cyclohexane at different concentrations (2.5, 5, 10, 15 % v/v) were added to the reaction mixture (conditions were as in 5.3). The mixture was incubated for 18 hours, and the production yield of cyclodextrins was determined. As shown in Figure 17, the production yield of total cyclodextrins and β-CD were

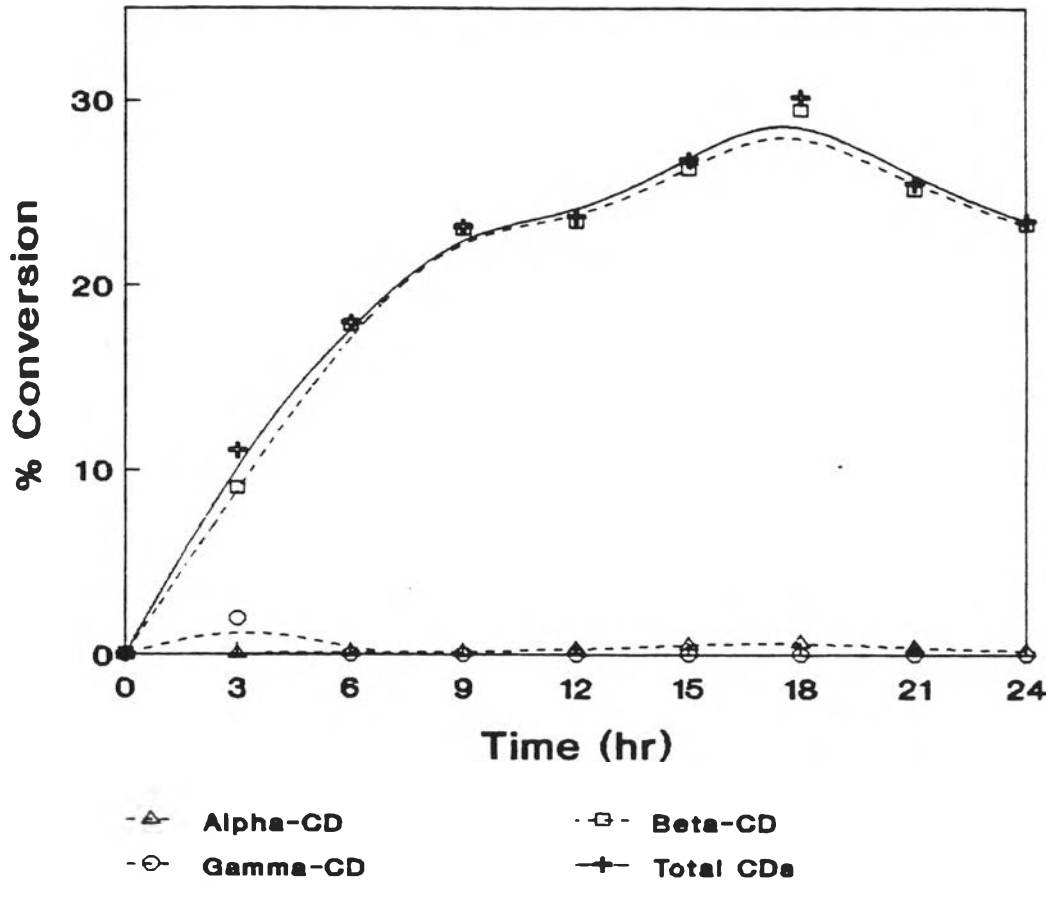


Figure 15 Time course of cyclodextrin production with complexing agent. Conditions were as described in Figure 13 except cyclohexane (5%,v/v) was added as the complexing agent as described in 3.3, Chapter II.

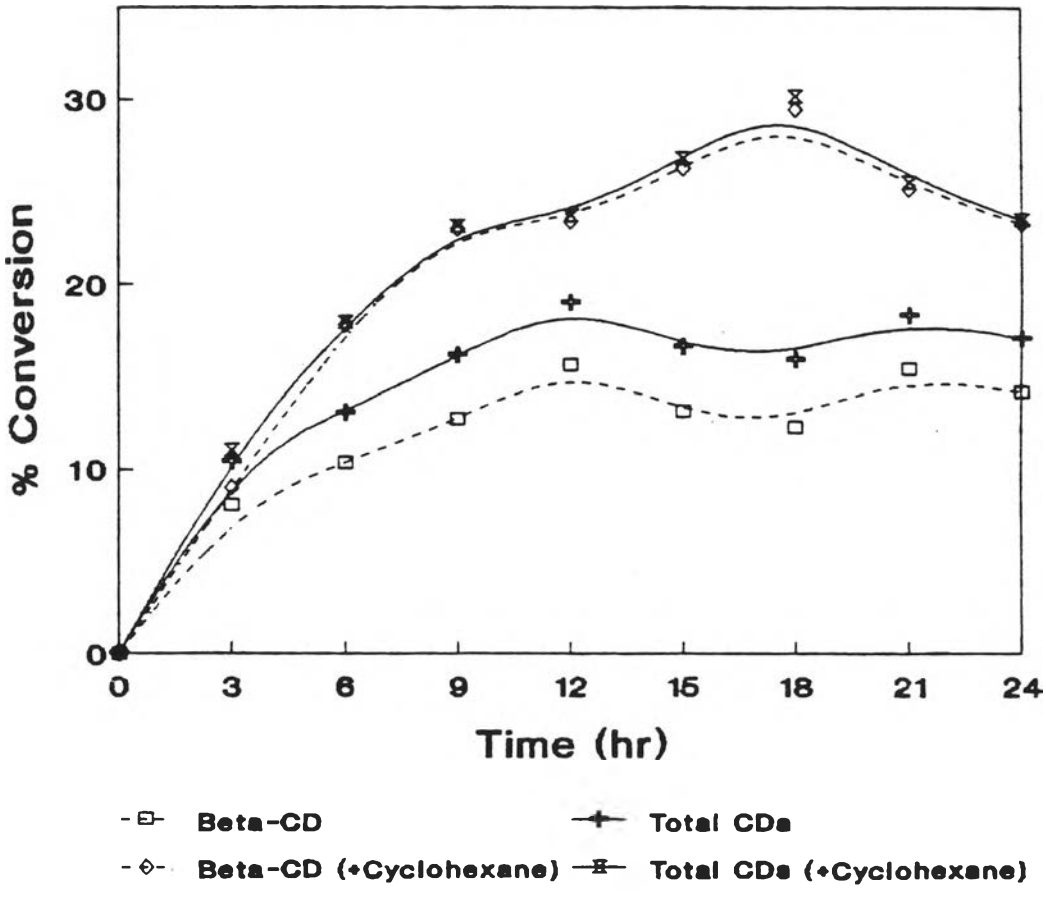


Figure 16 Time course of cyclodextrin production with/without cyclohexane.

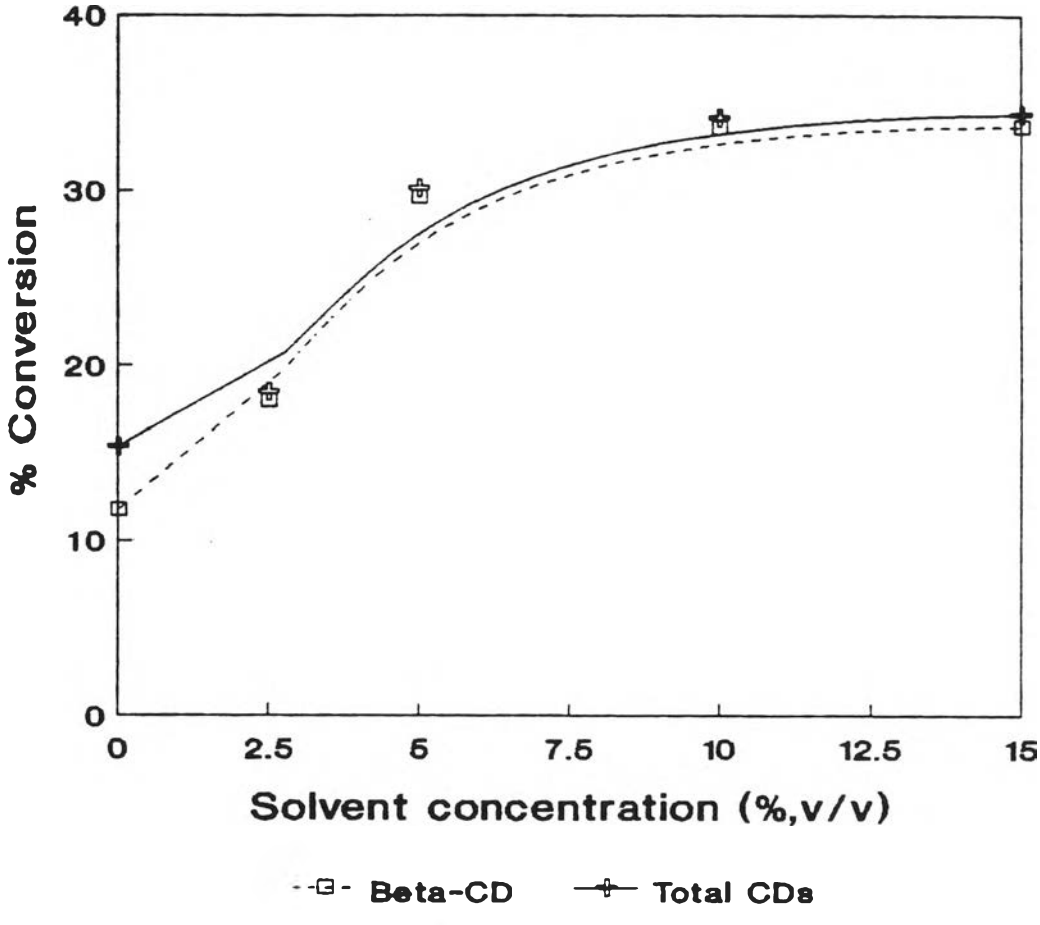


Figure 17 Effect of cyclohexane concentration on the production yield of cyclodextrins. Conditions were as described in Figure 15 except that reaction mixtures were incubated with different concentrations of cyclohexane for 18 hours.

rapidly increased and leveled off at around 5 % (v/v). Although there was an approximate 10 % increase in the yield at higher concentration of cyclohexane, lower concentration was preferable considering the toxic effect and the cost of removing such organic solvent. The appropriate cyclohexane concentration of 5 % (v/v) was selected to enhance the production yield of total cyclodextrins and β-CD.

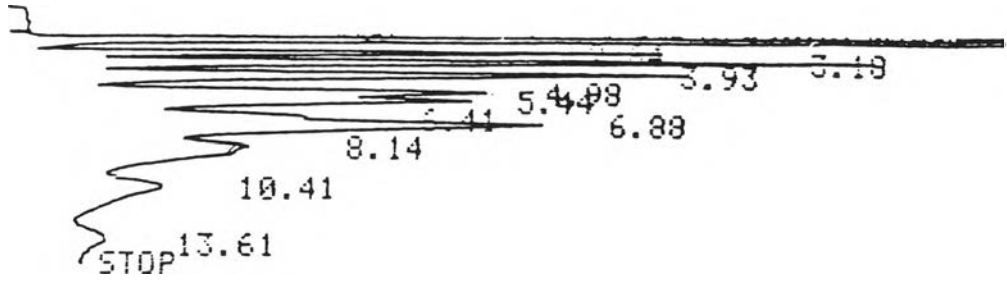
6. Product separation

6.1 Separation of cyclodextrins from non-cyclic products

The observed HPLC chromatogram of the reaction products occasionally showed an overlap between α-CD and G_4 peaks or a shoulder at the β- or γ-CD peak (Figure 18a) or a shift in retention time of all cyclodextrin peaks (Figure 18b). This indicated the presence of some linear oligosaccharides contaminated with cyclodextrin product in the reaction mixture. When HPLC patterns of some linear oligosaccharide standards were checked, it was found that non-cyclic oligosaccharides especially maltotetraose (G_4), maltopentaose (G_5) and maltohexaose (G_6) had retention times close to α-CD, β-CD, and γ-CD, respectively (Table 13).

From the example of HPLC chromatogram of reaction products shown in Figure 18b, it was difficult to identify whether the peak at the retention time of 5.08, was totally α-CD or not since it was in between those of pure maltotetraose (G_4) and α-CD. The same pattern was observed with the last two reaction product peaks (R_t = 6.66 and 8.46), which was in between peaks of pure β-CD and G_5, and pure γ-CD and G_6, respectively. It is very likely that the reaction products had some linear oligosaccharides contaminated with the cyclic products causing peak

(a)



(b)

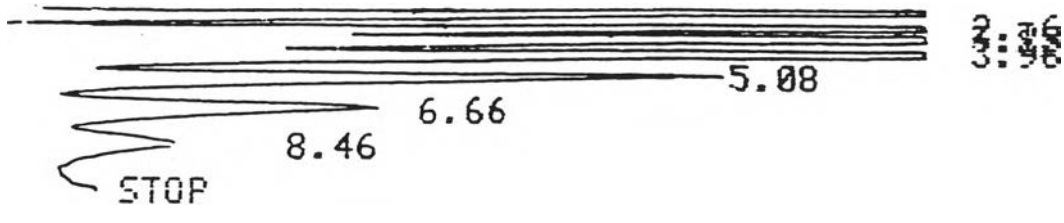


Figure 18 HPLC Chromatograms of the reaction products.

(a) an overlap or a shoulder at the CD peaks

(b) a shift in retention times of CD peaks

Table 13 Retention time of standard CDs and linear oligosaccharides on HPLC Supelco-NH_2 column. Conditions were as described in Section " Analysis of cyclodextrins ", Chapter II.

Standard	Retention time (min)
Glucose (G_1)	2.50
Maltose (G_2)	3.00
Maltotriose (G_3)	4.00
Maltotetraose (G_4)	4.90
Maltopentaose (G_5)	6.30
Maltohexaose (G_6)	8.20
α-CD	5.40
β-CD	6.70
γ-CD	8.90

overlap. The speculation was confirmed by adding either linear oligosaccharides (G_4, G_5, G_6) or CDs (α-, β-, γ-CD) as the internal standards.

In the attempt to differentiate the linear from the cyclic oligosaccharides, amyloglucosidase (AMG), and β-amylase were used. Standard oligosaccharides and cyclodextrins (5 mg/ml) were incubated either with 20-40 Units of amyloglucosidase for 1-2 hrs at 50°C, or with β-amylase at 25°C. The result showed that amyloglucosidase not only hydrolyzed linear oligosaccharides, but was able to destroy γ-CD even at 20 Units for an hour (Figure 19 and Table 14). The pattern shown in Figure 19 demonstrated the disappearance of peaks of G_4 (R_t = 5), G_5 (R_t = 6.41), G_6 (R_t = 8.19) and γ-CD (R_t = 8.7) which were completely hydrolyzed to glucose (Rt ≅ 2.6) by amyloglucosidase.

On the other hands, all cyclodextrins resisted β-amylase treatment while maltotetraose (G_4), maltopentaose (G_5), maltohexaose (G_6) were completely hydrolyzed to maltose (G_2) and maltotriose (G_3) (Figure 20). Table 15 showed that 20 Units of β-amylase at 25°C for 1 hr completely hydrolyzed the linear oligosaccharides (G_4-G_6) in the reaction products. In addition, it was found that 1/2 hr of β-amylase treatment was not enough to destroy these linear oligosaccharides, since the α-CD peak of the reaction products still showed a small overlapping of G_4 peak (data not shown), while extending the treatment time to 1 hr resulted in the homogeneous α-CD peak (Figure 20 : see profiles of reaction products before and after β-amylase treatment).

To determine the real amount of cyclodextrins produced, the reaction product after " Production Step" was treated with 20 Units of β-amylase at 25°C for

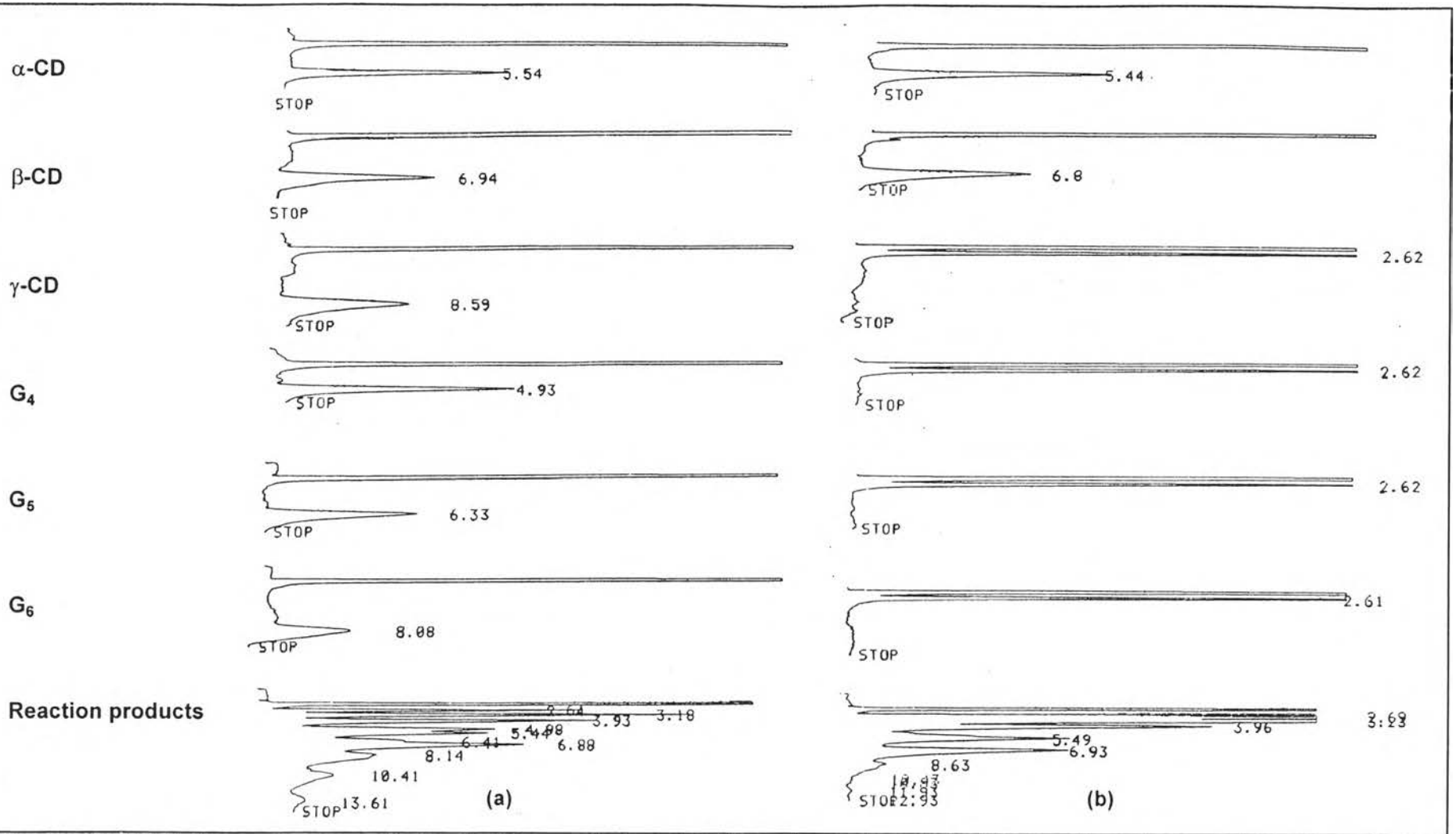


Figure 19 HPLC Chromatograms of standard linear oligosaccharides, standard cyclodextrins, and reaction products treated with amyloglucosidase. (a) Control (b) After AMG treatment (20 U, 50°C,1 hr)

Table 14 Treatment of reaction products with amyloglucosidase (AMG).

	Retention time (min) / Amount (%)			
	Control	AMG Treated		
		20 U, 1 hr	20 U, 2 hrs	40 U, 1 hr
Standard				
α-CD	5.54 (97.9)	5.44 (99.9)	5.44 (99.2)	5.48 (97.7)
β-CD	6.94 (99.1)	6.80 (98.0)	6.85 (88.0)	6.86 (92.9)
γ-CD	8.59 (97.8)	8.61 (3.2)	-	-
G_4	4.93 (98.2)	-	-	-
G_5	6.33 (99.9)	-	-	-
G_6	8.08 (83.0)	-	-	-
Reaction products				
α-CD	5.44 (11.0)	5.49 (8.2)	5.46 (6.3)	5.49 (6.2)
β-CD	6.88 (18.5)	6.93 (8.1)	6.95 (7.0)	6.94 (7.1)
γ-CD	8.14 (13.2)	8.63 (1.8)	8.61 (0.7)	8.64 (0.7)

- , peak was totally hydrolyzed

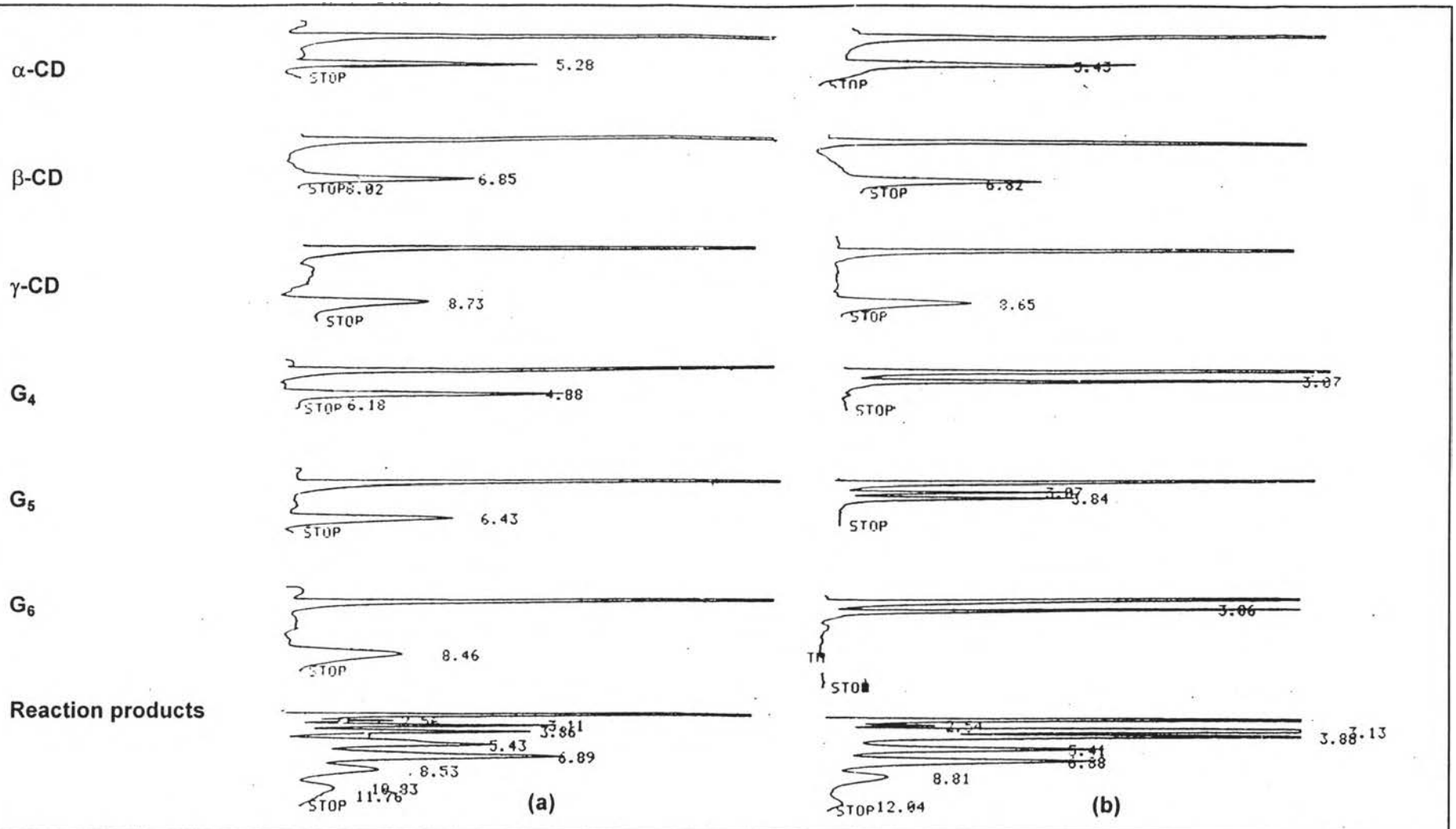


Figure 20 HPLC Chromatograms of standard linear oligosaccharides, standard cyclodextrins, and reaction products treated with β-amylase. (a) Control (b) After β-amylase treatment (20 U, 25°C, 1 hr)

Table 15 Treatment of reaction products with β-amylase.

	Retention time (min) / Amount (%)			
	Control	β-amylase Treated		
		20 U, 1 hr	20 U, 2 hrs	40 U, 1 hr
Standard				
α-CD	5.28 (95.4)	5.43 (99.5)	5.43 (99.0)	5.43 (95.4)
β-CD	6.85 (93.1)	6.82 (93.1)	6.82 (99.4)	6.83 (96.5)
γ-CD	8.73 (85.7)	8.65 (95.9)	8.52 (98.6)	8.73 (95.0)
G_4	4.88 (99.9)	4.83 (0.8)	4.86 (4.1)	-
G_5	6.43 (99.9)	6.49 (0.6)	-	6.46 (0.5)
G_6	8.46 (99.9)	8.51 (0.7)	-	8.43 (0.7)
Reaction products				
α-CD	5.43 (22.1)	5.41 (10.5)	5.42 (10.4)	5.40 (11.3)
β-CD	6.89 (31.3)	6.88 (12.4)	6.89 (13.3)	6.84 (12.9)
γ-CD	8.53 (13.1)	8.81 (4.2)	8.66 (3.7)	8.67 (4.9)

- , peak was totally hydrolyzed

an hour. The results shown in Section 2 - 5 were the values obtained after β-amylase treatment.

Table 16 summarized the result of cyclodextrin production at different conditions. With the use of complexing agent (cyclohexane), total cyclodextrins yield increased from 24 to 44 g%. However, after β-amylase treatment, the yields decreased to 19 and 34 g % in the production without and with complexing agent, respectively. The enzyme β-amylase thus hydrolyzed approximately 5-10 g % of the reaction products which were linear oligosaccharides (G_4-G_6) contaminated in both conditions of production ; with/without complexing agent conditions.

6.2 Separation of β-CD from cyclodextrin mixtures

In order to recover β-CD from the production with the use of complexing agent, the reaction products was evaporated to remove the complexing agent, concentrated (2/3 of volume), treated with β-amylase, and crystallized in water. Product crystallization was performed at 25°C or at 4°C for comparision (Figure 7). The crystalline material from each step was checked for its content by HPLC. The result showed that recrystallization was not necessary since only 1st crystallization could completely separate β-CD from α-CD (Table 17). Moreover, upon recrystallization, some amount of β-CD was dissolved in Filtrate 2 which unnecessarily made the amount of recrystalline lower. Additionally, crystallization at 4°C yielded twice the amount of β-CD obtained from crystallization at 25°C (Table 17,18). The HPLC patterns of Filtrate 1 and 1st crystalline at 4°C were compared (Figure 21). Filtrate 1 consisted of 42 % β-CD, 4 % α-CD and 51 % G_1 -G_3 , while 95 % of the constituents in the crystalline was β-CD with 5 % G_2 -G_3.

The recovery of β-CD in 95 % purity was approximately 40 % of β-CD obtained from production prior to crystallization. The remaining 60 % in Filtrate 1 which could be harvested by further concentration and crystallization, if desirable. The final amount of pure β-CD crystal prepared from 100 g of rice starch as the starting material in this work was calculated to be 12.48 g.

Table 16 Yield of cyclodextrins at different conditions.

Condition	Yield of cyclodextrins (g %)			
	α-CD	β-CD	γ-CD	Total CDs
Without Complexing agent	1.29	19.10	3.39	23.78
Without Complexing agent (+ β-amylase)	0.84	15.59	2.56	18.99
With Cyclohexane	2.04	42.21	-	44.25
With Cyclohexane (+β-amylase)	0.32	33.83	-	34.15

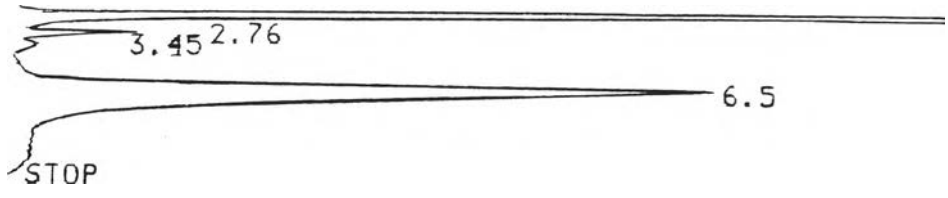
Table 17 Yields of cyclodextrins after crystallization at 4°C.

	Yield of cyclodextrins (g %)		
	α-CD	β-CD	γ-CD
Filtrate 1	0.93	18.04	-
Crystalline	-	12.48	-
Filtrate 2	-	3.66	-
Recrystalline	-	8.75	-

Table 18 Yields of cyclodextrins after crystallization at 25°C.

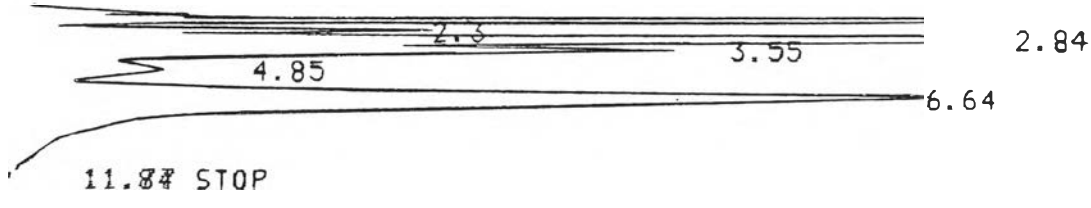
	Yield of cyclodextrins (g %)		
	α-CD	β-CD	γ-CD
Filtrate 1	0.71	25.28	-
Crystalline	-	6.31	-
Filtrate 2	-	4.52	-
Recrystalline	-	0.42	-

(a)



#	NAME	TIME	CONC	MK	AREA
0		2.76	4.0187		37277
0		3.45	0.7436		6897
0		6.5	95.2376	v	883402
		TOTAL	99.9999		927576

(b)



#	NAME	TIME	CONC	MK	AREA
0		2.3	3.7122		99780
0		2.84	35.6867	v	959223
0		3.55	13.6306	v	366378
0		4.85	4.108	v	110419
0		6.64	42.8623	v	1152094
		TOTAL	100		2687895

Figure 21 HPLC Chromatograms of cyclodextrin products after 1st crystallization compared to Filtrate 1.

(a) Crystalline (b) Filtrate 1