CHAPTER IV

DISCUSSION

In the initial phase of study, gelatinized cassava starch was used as substrate of CGTase from *Bacillus circulans* A11 using standard condition (2 g% starch, 500 U/g starch,17 hours and 40°C), the total conversion and ratio of α: β: γ -CD were 37% and 2.8:3.7:1 respectively. When compared with other starches, it showed that cassava starch was a good substrate for cyclodextrin production as well as amylopectin (corn), rice (long grain) and Thai glutinous rice. When compared with corn and potato starch which were used in commercial cyclodextrin production, cassava starch produced higher yield of total CDs than corn starch (34%) but lower than that of potato starch (42%) (Figure. 11). The results also indicated that many kind of cereal starches were good candidate for cyclodextrin production except amylose (corn) and rye which gave the lowest yield of 28%. Notably, sago starch gave very high conversion yield of 43%. However, the milling process for sago starch is difficult and is import starch in Thailand.

Since amylose and amylopectin are the main substituent in starch, the relationship between these polysaccharides content and cyclodextrin yields were investigated. The result from the use of amylose and amylopectin (Sigma) clearly showed that amylopectin was a better substrate than amylose (Table 7). When Thai glutinous rice which has high amylopectin content similar to amylopectin sample from Sigma (~92%) also gave high and similar amount of total CD. Rendleman (2000) studied the hydrolytic action of α-amylase on native and cooked starch. His observation indicated that high amylopectin starches tend to become more susceptible to α-amylase than high amylose starch. Rendleman also stated that dissolved or gelatinized form of starches react with α-amylase more readily. The hydrolytic rate on raw or native granular starches varied according to the source of starch. Since CGTase has similar amylolytic activity (disproportionation and / or coupling reaction) and 3 out of 5 domains

of the CGTase molecule are the same as α-amylase molecule, similar amylolytic results might be applied for both enzymes.

However, it could not be concluded that there was a correlation between amylose/amylopectin content and CD yields from Table 7 since contradictory result also occurred. Corn and short grain rice differed about 10% in amylopectin content but they yielded the same amount of CD. On the contrary, short grain rice and rye had about the same amylopectin content (~86%) but their CD products are 34 and 27% respectively. Cassava (starch) which contained relatively the same amount of amylopectin as rye (83% vs 85%) produced 10% more of CD than rye.

It is my opinion that the production yield of CDs could not be attributed to, apparent form, the % content of amylose or amylopectin alone. There are many more factors that may affect the reaction of CGTase with the polysaccharides. The fine structure of amylose and amylopectin within the starch granules should be taken into consideration. Oates (1997) gave an excellent review on this subject allthough he felt there is still many features remained unaddressed. Amylopectin in starch granule are arranged in cluster structures, which composed of amorphous and crystalline regions. The crytalline polymorph can be devided into A, B or C polymorph according to the packing configuration of the branch chain to form a unit cell structure. Amylose molecules are bundled between the amylopectin clusters within both the amorphous and crystalline regions. In his review, Oates claimed that the different crystal structures account for some of the variation between different starch types. Studied on the hydrolysis of raw starch from various sources by α-amylase, he showed that the type of crystal polymorh of the starches correlated more to amylose susceptibility (A>C>B) than their % amylose content.

In addition to the afore metioned factors, other properties of the starch should also taken into consideration. Besides the different granules morphology and mode of enzyme attack for each kind of starch (Oates, 1997; Yamamoto et al., 2000). Oates (1997) suggested that the presence of proteins and lipid may influence the susceptibility

of granules to enzyme hydrolysis e.g., influence on the adsorption of starch granules to enzymes. In the study of Figure 11 and Table 7, the polysaccharides used could be devided into 2 groups: starch (amylose (corn), amylopectin (corn), potato, sago and cassava) and flour (rice (long grain), rice (short grain), Thai glutinous rice, corn, wheat, arrow root and rye). Bennion (1980) reported the protein content of wheat in ranged of 7.5-13.3% whereas the lipid content in short grain rice was 0.63-1.11%. The samples used in this thesis were not determined for both moieties so the affect of protein or lipid to CGTase was not considered.

Last, but not least, the physical properties of the starch (which in turn reflect from the chemical property of the molecule) could account for the different CD yield from different kind of starch. Its solubility, viscosity in suspension, retrogradation property, and swelling power could affect the interaction of CGTase with starch molecules. The viscosity of 5 g% raw starches/flours in distilled water (used in Figure 11) was shown in Appendix L. Since subsequent studies for CD production in this thesis used gelatinized and enzymatic pretreated starch, and the concentration of starch is not high (2.5 g%); therefore viscosity should not be a limiting factor here.

To calibrate optimal condition for CD production from cassava starch, the effect of starch concentration, time and tempervalue of incubation, and enzyme concentration was studied. 1 g% of starch gave the highest conversion yield of 47% by CGTase from *Bacillus circulans* A11 (Figure 16). When the starch concentration was increased, the total yield of CDs was decreased. Bergsma et al., (1988) reported that the yield of cyclodextrin from potato starch and CGTase from *Bacillus circulans* decreased from 43 to 19% when potato starch concentration was increased from 1 to 22 g%. Similar observation was reported by Yim et al., (1997), using soluble starch incubated with CGTase from *Bacillus firmus*. The reduction of CD products might be due to the presence of small sugar units in the reaction mixture. Lee and Tao (1995) reported that small molecules of maltooligosaccharides which were produced during reaction can strongly inhibit the cyclization reaction. Influence of saccharides as inhibitors of CD production was extensively reported by Rendleman (1996). Removal of small

saccharides and end products (CDs) are recommended and routinely used in commercial process.

The optimum temperature for cyclodextrin production was 40-45°C (Figure 17). These results coincided with previous studies of Techaiyakul (1991) and Kaskangam (1998). Kaskangam also found that there are 4 isoforms of CGTase from *Bacillus circulans* A11 with optimum temperature of 40 °C, 40 °C, 50 °C and 50-60 °C. Sato and Yagi (1991) demonstrated that CGTase from *Bacillus circulans* had optimum temperature between 40-60°C. From these results, To lower energy cost, all subsequent experiments were conducted at 40 °C.

Studied on the effect of enzyme concentration and incubation time on the total yield and ratio of cyclodextrins showed that these two factors affected on the kind of cyclodextrins produced. At enzyme concentration 500 U/g starch gave the highest total yielded (~35%) and the yield of α-, β-, γ-CD was 1.3:3.8:1 (Figure 18). When the enzyme concentration was increased, the α - CD was also increased and reached maximum at 1250 U/g starch. On the contrary the report by Sato and Yagi (1991) showed that the cyclodextrin ratio was not changed at various concentrations of CGTase from *Bacillus circulans* IFO3329. When CGTase concentration was increased, the total CDs yield was changed. The discrepancy may be due to the different conditions such as incubation temperature (50°C), incubation time (24 hours) and substrate (soluble starch) used. At enzyme concentration of 1250 U/g starch, the amount of α - CD was the highest (13%) and was comparable with β-CD. The ratio of α: β: γ-CD at 2.96:3.29:1. Moreover, Figure 18 showed that at all enzyme concentration, the content of gamma CD obtained low constant level. At low enzyme concentrations (25-250U/g starch), there were more γ-CD than α-CD although β-CD was the majpr product at all enzyme concentration. Malai (1995) reported that at low amount of CGTase from *Bacillus circulans* A11 (<200 U/g starch), the β-CD was the major product when incubated with 10 g% liquefied rice starch for 17 hours.

The effect of incubation time was studied as shown in Figure 19 and 20. At CGTase concentration of 500 U/g starch, with prolonged incubation time, the ratio of α: β :γ-CD was changed from 0.09:2.7:1.6 at 1 hour to 1.3: 2.7:1 at 24 hours. The highest yield of total CDs was 8 hours. At CGTase concentration of 1250 U/g starch, with prolonged incubation time, the ratio of α: β: γ-CD was changed from 2:3:1 at 1 hour to 14:12:1 at 48 hours. The highest yield of total CDs was 16 hours. These results disagreed with a previous report of Stominska and Sobkowiak (1997) experiment using CGTase from *Clostridium*. They reported that the prolonged reaction time influence slightly the decreased amount of α-CD and the increased amount of β-CD.

The conventional method of industrial cyclodextrin production used high starch concentration (10-30%) which caused high viscosity of the suspension. Stirring of the suspension for homogeneity with enzyme was difficult. In order to solve this problem, the starch is firstly preheated by heating and/or enzyme hydrolysis so that starch paste liquefied and the polymeric chains are shortened. Such pretreated starch was the appropriate substrate for the next step of CGTase catalysis. Generally, α-amylase was used for the liquefaction step. Figure 21 showed that total CD obtained from 5g% cassava starch liquefied with α-amylase of 0.0024-0.012 U/g starch for 20 minutes was more than that from non-liquefied starch (32% vs 28%). Increasing α-amylase concentration decreased the total yield. Pullulanase, which cleaves α(1-6) glycosidic linkage was another liquefied enzyme employed. In this case, the amylopectin branches were destroyed at least partially, reduced the paking of amylopectin unit cells and become easier for enzyme hydrolysis (Bertoft and Kock, 2000). Pretreatment of gelatinized starch, which pullulanase $\geq$ 48 U/g starch increased the total CD yield from 28 % to 37%(Figure 22). This result agreed with that of Rendleman (1997), using *Bacillus marcerans* CGTase. Yield enhancement was even more when Rendleman used a combination of GCTase and complexant. If α-amylase and pullulanase were used simultaneously to liquefied cassava starch, enhancement of CD production was greater than control, using α-amylase alone, or using only pullulanase 24-48 U/g starch (Table 9). The best combination of enzymes would be 96 U pullulanase and 0.0024 – 0.024 U α-amylase per gram starch. Treatment of starch with pullulanase and maltogenase or

fungamyl prior to CGTase gave very low CD yield (10-12 %). These two enzymes catalyze the cleavage of starch into maltose (average DP~2, Table 9). Small oligosaccharides (DP<4) will favor coupling reaction (Kitahata et al., 1978; Rattapat, 1996; Bender, 1982). Kitahata and Okada (1975) reported that glucose and maltose were effective acceptors for disproportionation reaction. Increasing the rate of these two reactions therefore would reduce the cyclization reaction of CGTase. Bender (1980) reported that substrate with chain length between 16-80 glucose units was good substrate for cyclization. Vetter and Thorn (1992) using demonstrated that DP 9 gave the highest total yields compared with other DP. Longer or shorter substrate than those could involve with other reactions of CGTase. The result from Table 9 indicated that pretreatment of cassava starch with α-amylase which yielded maltodextrin of average DP 16-66 gave higher % CD than control. Shorter chain length resulted in lower CD product. Addition of pullulanase to amylase resulted in both chains debranching and chain shortening, giving average DP of 5-19. They were better substarte for cyclyzation by CGTase as obvious from the higher increased % CD. Suprisingly, treatment of 96 U pullulanase per gram starch yielded maltodextrin of average DP 100 still gave high CD yield (37%) although Bender (1980) suggested that chain length greater than 100 glucose unit flavoured disproportionation reaction. It should be stressed that the value of DP reported here was the average DP from a mix population of starch hydrolysate. Also, CGTase contains disproportionation quality, which can help tailoring starch chain into appropriate chain length for cyclyzation.

To obtain more understanding on the relationship of chain length on cyclodextrin production, cassava starch was incubated with either α-amylase or pullulanase, ultrafiltrated (M.W.cut off 30,000), and the hydrolyzed cassava starch using α-amylase was filtrated by ultrafiltration (cut off 30,000). The hydrolyzed starch was fractionated by Biogel P-10 column (fraction range 1,500-20,000). It was found that cassava starch liquefied with α-amylase gave mainly DP < 20 oligosaccharides (Figure 26). Highest CD yield (37%) was obtained from pooled fraction with average DP 9 (Figure 27). This finding agreed with previous study of Bender (1990) and Vetter and Thom (1992). Moreover, it was found that the average DP 4 fraction gave higher content of γ-CD than

the other cyclic polymers with ratio of α:β:γ-CD 8.71:1:15.78. This finding corresponded with a previous study of Rattapat (1996) who observed high proportion of γ-CD when 2 g% of pure maltotetraose or maltoheptaose was incubated with 50 U CGTase from *Bacillus circulans* A11 for 24 hours. Figure 27 also demonstrated that DP>20 fractions were not suitable for γ-CD production. Fraction with average DP of 9 gave highest yields of α and β-CD. In fractions of DP 20-28, α-CD was more than β-CD. Strokopytov and colleagues (1996) demonstrated that the active site of CGTase could bind at least 9 glucose. Each sugar binding site was called subsite. Each subsite had differenct binding energy to sugar molecule due to the distortion of sugar chains. Hence, the size of maltooligossaccharides may affect the kind of CD product (Uitdenhagg et al., 2000). It may be concluded that the size of oligosaccharides chain affected on the kind of CD produced.

Cassava starch liquefied with pullulanase was fractionated into 3 fractions by Biogel P-10 column. It was shown that fractions of lower DP gave higher cyclodextrin yield (Figure 29) similar to that using α-amylase. Ranks of increasing production yield were DP 26>DP 75>DP id and DP 75 fraction gave more α-CD than β-CD.

However, it is not necessary that cassava starch of same DP value from liquefraction by α-amylase or pullulanase should give the same yield due to the different action of enzymes. The result in Table 9 supported this conclusion.

Using partially purified DP 9, 26 and 75 fractions as substrate for varying CGTase or incubation time, it was clearly shown that enzyme concentration and reaction time could influence type and amount of CD products. Stominska and Sobkowiak (1997) also reported the same observation.

Generally, addition of organic solvent was used to enhance production of cyclodextrin (French et al., 1949) or to purify certain cyclodextrins from mixture (Bender, 1986). Most organic solvents added to reaction mixture were alcohol, polyalcohol or dextrin and many showed complexing specificity to type of CD (Shiraishi, et al., 1998;

Rendteman, 1997). The result in Figure 33 showed that ethanol promoted mainly β-CD. At above 20% (v/v) ethanol, total CDs yield decreased possibly because of the denaturation of the enzyme (Shiraishi, et al., 1989; Mattsson et al., 1992). Using 2.5 g% starch, 500 U CGTase/g starch in the presence of 20% ethanol, it was shown that total CDs would increase with longer incubation period, reaching maximum at 36 hours (Figure 34), and the β-CD yield increased 69.6% when compared with control. Shairaishi et al., (1989) studied on the effect of ethanol on *Bacillus macerans* CGTase and postulated that the enchancement of CD production by ethanol might arise from 3 possibilities. 1) the formation of an inclusion complex with CD and thus reduction of end products inhibition; 2) reduction of water activity there by increase hydrolytic reaction; and 3) support the binding reaction between enzyme and substrate. It was probable that when alcoholic complexants were added, a change in the conformation of CGTase and substrate may take place because these complexants reduced dielectric constant of reaction. The molecules may bind to specific sites on the enzyme and inhibited the hydrolytic reaction. Mattsson et al., (1992) reported that the binding of CGTase to starch was considerably increased by adding 10-20% ethanol. This could be due to a change of k_m, general solubility effects or the enhanced surface area on starch. Lee and Kim (1991) reported the enhancement of β-CD yield was about 2 times when the incubation mixture contained ethanol 10 %(v/v), corn starch and alkalophilic *Bacillus* sp. CGTase. In contrast Shiraishi et al. (1989), they showed that when soluble starch was incubated with CGTase from *Bacillus macerans* in the presence of (10%, v/v) ethanol for 24 hours, the increase of α-CD was 1.8-1.9 times. These differences in results may be related to the kind of substrate and type of CGTase used.

The effects of some other aliphatic alcohols and CGTase concentration on cyclodextrin production were also determined. In order to compare the result with that of ethanol; 20% (v/v) of complexant was employed and the reaction time was kept at 36 hours. At CGTase concentration of 50 U/g starch, the amount of β- and γ-CD was more than α-CD in the absence of complexant (α:β:γ 1:32:10) (Table 10). Again, it was apparent that all complexants used could enchance total CD yield. Only the extent and α:β:γ CD ratio varied.

When alcoholic complexants were added to the reaction mixture, the amount of the β-and γ-CD was increased. It was remarkable that γ-CD was increased 1.5-2 times even in the presence of 20% complexant as contrast to the result from 500 U CGTase (Figure 33). The effect on α-CD was inconsistant but a position trend was indicated except for methanol. At CGTase concentration of 500 U/g starch, the amount of α-and β-CD was more than γ-CD in the absence of complexant (10:17:5) (Table 10). When alcoholic complexants were added to the reaction mixture, the amount of α-CD was markedly whereas decreased β-CD was increased. At these enzyme and complexant concentrations, γ-CD was not produced with the exception of methanol. Total CDs and β-CD yield were highest in the presence of 2-butanol. When CGTase concentration was raised to 1500 U/g starch, only α-and β-CD were produced in the absence of complexant. When compared with results from Figure 20 which showed that at CGTase 1,250 U/g starch, initial phase of reaction gave three kinds of CDs but with prolonged incubation time (>18 hours), only α- and β-CD are produced. Therefore, the result in Table 10, using CGTase 1500 U/g starch and 36 hours incubation period, no γ-CD should be observed. When alcoholic complexants were added to the 1,500 U CGTase reaction mixture, again, the amount of α-CD was decreased and β-CD was increased with the except of methanol.

It could be concluded from these results that enzyme concentration, incubation time and type alcoholic of complexant affect on the total CDs yield and the ratio of α-,β- and γ-CD. However, 1-butanol and 2-butanol were very good complexant. This result coincided with a previous study by Rohrbach et al., (1988). The results showed that β-CD yield was increased 2 times when 2-butanol 5%(v/v) was used. This result disagreed with previous study by Lee and Kim (1992), who reported that 1-butanol and 2-butanol decreased the total yield. It may be due to different condition used such as type of substrates (corn) and enzymes. Rohrbach et al., (1988) reported that the effect of the alcohol depended on both the nature of alcohol and its concentration. However, the selection of complexant depended on price, toxicity and from the crystalline end product. Using enzymatic pretreated cassava starch (2.5 g%) and incubate period of 36

hours, the suitable conditions are as follows: For α-CD, 500 U of CGTase in the presence of 20 %(v/v) ethanol or 1,500 U of CGTase without addition of complexant could be chosen. For β-CD production was 2.5 g% cassava starch incubated with CGTase 500 U/g starch and 2-butanol (20%, v/v) as complexant for 36 hours. For the γ-CD production, the condition should be 2.5 g% cassava starch, 50 U CGTase in the presence of 20% (v/v) ethanol. Although γ-CD could be produced with methanol under higher concentration of CGTase, methanol is a highly toxic compound and should be avoided.