CHAPTER IV

DISCUSSION

In the production of cyclodextrins which have been reported so far, corn or potato starch was generally used as the starting material (Bender, 1986). Since rice starch is the country 's main agricultural product, it is of interest to develop an effective process for cyclodextrin production from rice starch. When considering the physical and chemical properties of common starches (Smith, 1982) (Table 19), it was found that rice starch has about 17 % amylose with the remainder being amylopectin. In comparison with potato starch, rice starch has amylose content relatively close to potato starch. However, their granule size and shape are different and the gelatinization temperature of rice starch is somewhat higher than potato starch. It seems that although their amylose contents are similar, the physical properties of the two types of starch differ. On the other hands, rice and corn starch, differ in both amylose contents and the granule sizes. Therefore, the differences in physical and chemical properties of starches might contribute to the action with CGTase enzyme. It has been reported that different types of starch affect cyclodextrin formation. Conversion of corn, potato, wheat, rice and waxy maize starches by CGTase of the *Thermoanaerobacter* ATCC 53627 gave cyclodextrin yield of 28.2 %, 29.5 %, 25.6 %, 17.7 % and 23.5 %, respectively (Starnes, Flint and Katkocin, 1990). Moreover, the different ability of CGTase in catalyzing carbohydrate substrates with various degree of polymerization has been reported (Nakamura and Horikoshi, 1976). The CGTase from alkalophilic *Bacillus* sp. (ATCC 21783) catalyzed 73 % of potato starch, 65 % of amylopectin,

Table 19 Physical and chemical properties of common starches. (Smith, 1982)

Starch	Granule size (mμ)	Granule shape	Amylose (%)	Gelatinization range (°C)
Corn	20	Round, polygonal	25	62-72
Waxy corn	20	Round, polygonal	0-3	63-72
Potato	35	Oval	20	56-69
Tapioca	18	Truncated, round, Oval	17	52-64
Sago	25	Oval, truncated	27	60-72
Wheat	10	Oval, round	25	62-75
Rice	7	Polygonal	17	61-78

45% of oyster glycogen and 25 % of amylopectin β-limit dextrin at 1 % (w/v) of substrate concentration.

Our group had earlier reported the development of the use of local starches in culturing medium of *Bacillus* sp. A 11 for enzyme CGTase and cyclodextrins production. Growth of *Bacillus* sp. A 11 and its CGTase activity when cultured in our modified Horikoshi's medium containing corn or tapioca or rice starch in replacement of soluble potato starch were studied. (Cultivation was carried out in a 5 l-fermenter at 36±2°C for 3 days. Aeration rate was 0.67 vvm and agitation speed was at 300 rpm) (Rutchtorn, 1993). The result showed that when commercial soluble starch was replaced by local rice starch, an increase in CGTase activity was observed. Moreover, comparing rice, corn, and tapioca starch, the highest CGTase production was observed in the rice starch-containing medium (Table 20). Quantitative analysis of cyclodextrins produced in the culture medium showed β-CD as the major product, with rice starch giving better yield than tapioca and corn starch (Table 21). We feel that rice starch should also be a good candidate among local starches to be used as the starting material for cyclodextrin production in the present system using isolated CGTase.

In the initial phase of present study, rice starch was used as substrate of CGTase isolated from *Bacillus* sp. A 11 in comparison with imported soluble potato starch which is commercially available. From our preliminary investigation, it was found that soluble starch yielded somewhat higher cyclodextrin products than rice starch. This may be due to the difference in their physical properties especially the inappropriate size or shape of polysaccharide molecules of rice starch which makes the reaction between the starch and the enzyme CGTase less effective. If the rice starch was pretreated by heating or by hydrolytic enzymes under optimum conditions

Table 20 Growth of *Bacillus* sp. A 11 and CGTase activities when grown in culturing medium containing 1.0 % of different types of starch. (Rutchtorn, 1993)

Starch	Lag phase (hr)	Maximum activity obtained		
		Dext. act.* (U/ml)	Specific act.** (U/mg)	CD-TCE (dilution limit)
Rice	23	136	269	2^{10}
Corn	25	78	167	2^{8}
Tapioca	16	64	180	2^{8}

* Dext. act. = Dextrinizing activity

** Specific act. = Specific activity

Table 21 The amount of β-cyclodextrin produced by *Bacillus* sp. A 11 in culture mediums containing various types of starch. (Rutchtorn, 1993)

Starch in the medium	β-CD produced (mg/ml enzyme)
Rice	14.5
Corn	9.5
Tapioca	12.0

to give suitable size/shape or characters of polysaccharides, it could be better substrate for the enzyme CGTase and yielded higher cyclodextrins.

In the commercial production of cyclodextrins, two different approaches have been used (Bender, 1986). One approach is the straight forward CGTase conversion of starch. The alternative approach involves the conversion of starch in the presence of suitable organic solvent precipitants. The study of cyclodextrins production with / without the use of organic solvent will be discussed in this present study.

Production of cyclodextrins without the use of complexing agent

In a conventional process of pretreatment step, raw starch is usually liquefied by heating or treatment with hydrolyzing enzyme, then cyclodextrin glycosyltransferase (CGTase) is added to convert liquefied starch to cyclodextrins (Lee and Kim, 1991). Generally, hydrolyzing enzyme used in the pretreatment step is α-amylase. CGTase was also reported to be used since it can degrade starch by disproportionation reactions. A thermostable CGTase which can withstand high temperature of pretreatment step was employed for starch liquefaction with the consecutive enzymatic conversion of liquefied starch to cyclodextrins (Starnes, Flint and Katkocin, 1990). For example, Chinoin Pharmaceutical Industries' patent uses α-amylase to liquefy starch before incubating with CGTase. While Nihon Shokuhin Kako Co.'s patent uses CGTase as the pretreatment enzyme. Our investigation demonstrated that rice starch, when used in high concentration, had to be pretreated in order to serve as the proper substrate for CGTase (Table 9). Pretreatment of starch in the presence of hydrolyzing enzyme, α-amylase was chosen. The optimum

concentration of α-amylase and the incubation time were investigated (Figure 8, 10). The appropriate pretreatment conditions were 0.1 % α-amylase at 50°C for 15 minutes. Treatment with hydrolyzing enzyme either increased solubility of starch or reduced the viscosity of starch solution. The main soluble products of starch digested by α-amylase were glucose, maltose and higher oligosaccharides which were further converted to cyclodextrins by the enzyme CGTase. We found that Dextrose Equivalent (DE) of starch should be in the range of 6-16 because if treated starch has too high DE value, it may not be suitable for being the substrate of CGTase. Moreover, treated starch with high DE has high concentration of short chain-maltooligosaccharides or glucose which renders the reaction of CGTase in favor of reversed coupling reaction (Kitahata, Okada and Fukai, 1978). It had been reported that excessive hydrolysis affects the cyclodextrin yields. Liquefied starch with DE values of 1-12 yielded 15.6-29.3 % of cyclodextrins while at DE of 23, only 5.2 % was obtained (Starnes, Flint and Katkocin, 1990).

The optimum starch concentration represents a compromise of several factors. As starch is an inexpensive raw material, the production of cyclodextrins at high concentration of starch is desirable from the economic standpoint, while the percentage yield is also an important factor to be considered. In this work, rice starch varied from 1-30 % (w/v) was used for cyclodextrin production. Rice starch was pretreated with α-amylase (conditions as described earlier), then incubated with CGTase (500 Units/g starch). As shown in Figure 11, the total amount of cyclodextrins produced increased with increasing concentrations of rice starch, but % conversion or production yield decreased. Ten g% was chosen in consideration of the yield and total amount of cyclodextrins obtained. Potato starch (6-20 g%) and corn starch (15-28 g%) had been used in other laboratories as starting materials

for large scale production of cyclodextrins (Bender, 1986).

The results in Table 11 showed that at low starch concentrations (0.5-5 g%) α-CD was the main product while at higher starch concentrations (10-30 g %), β-CD was observed to be the major CD forms. The ratio of α : β : γ shifted from 6.2 : 4.4 : 1 to 1.6 : 2.3 : 1. Similar observation was also reported when corn starch was converted to cyclodextrins by *Thermoanaerobacter* ATCC 53627 CGTase (Starnes, Flint and Katkocin, 1990). Thus, selective production of cyclodextrin of interest could be performed by choosing the right concentration of starch.

To test for the influence of CGTase concentration on the yield of cyclodextrins, treated rice starch was incubated with various amounts of enzyme (15-1000 Units/g starch). It was shown that only 25 Units CGTase/g starch was enough for maximum cyclodextrin production (Figure 12). The optimum CGTase : substrate ratio (w/w) in this study was 1 : 100,000. It had been earlier reported that E : S ratios (w/w) of 1 : 1,000 - 1 : 5,000 (Horikoshi *et al*, 1982 ; Bender, 1983, 1984) gave best result for industrial cyclodextrin production. That different ratio of E : S which gave different products was demonstrated in this study. 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. Again, preference production of certain cyclodextrin could be performed, this time by varying the concentrations of CGTase. This together with the discussion in the previous paragraph, indicated the importance of the ratio of starch substrate to CGTase enzyme (either by varying starch or CGTase) on the selective production of cyclodextrin of interest.

In conclusion, production of cyclodextrins from rice starch (10 g %) by

CGTase of *Bacillus* sp. A 11 yielded 24 g % crude CDs or 19 g % pure CDs (15.6 g % β-CD). The production obtained was comparable to the Nihon Shokuhin Kako's patent which converted potato starch (15 g %) using CGTase of the alkalophilic *Bacillus* 38-2 and 19 g % β-CD was obtained.

Production of cyclodextrins with the use of complexing agent

Cyclodextrins were reported to be processed from starchy substrate in the presence of an appropriate solvent molecule, where the applied solvent forms an insoluble inclusion complex with cyclodextrins (Seres and Barcza, 1988). The selectivity of complex formation depends on various parameters. Such parameters like geometrical fit and apolarity of the guest would be critical in formation with individual cyclodextrin. The use of such organic solvent, namely the complexing agent, was generally reported to improve the cyclodextrin yield or to select certain type of cyclodextrin (Seres and Barcza, 1988). Either one or two solvent system had been used which resulted in the formation of binary or ternary complex with the cyclodextrin host. A ternary complexation process may result in more stable cyclodextrin complexes due to the better space filling of paired guests and higher stability constant of the resulting ternary complex molecule (Seres and Barcza, 1988). For example, the methyl-ethyl ketone (MEK)-sodium dodecyl sulphate (SDS) guest pair was proven to form complex with α-CD, the MEK-toluene pair was good for β-CD complex formation, and MEK-naphthol or MEK-xylenol isomer guest systems were efficient for the formation of γ-CD complex (Seres and Barcza, 1988).

In the present work, cyclohexane, trichloroethylene (TCE) , toluene, naphthalein (Nap), n-decyl alcohol, ethanol, methyl-ethyl ketone (MEK) were used

for the binary complex formation system (such as a cyclohexane-CD binary complex). While Nap-MEK and cyclohexane-MEK were used for the ternary complex system study (such as Nap-CD-MEK ternary complex). According to our preliminary check (5.1, Chapter III), the dextrinizing activity of CGTase from *Bacillus* sp. A 11 was not changed by all complexing agents tried under the described conditions except for n-decyl alcohol. However, all complexing agents including n-decyl alcohol were further used in comparison in the production of cyclodextrins.

By using single solvent system, cyclohexane, trichloroethylene, and toluene were very good complexing agents since they increased production yield from approximately 16 g% to 34 g%. Cyclohexane was selected as the solvent of choice since it gave high yield at lower CGTase activity when compared to trichloroethylene and toluene (Table 12). Additional reasons contributed to the selection of cyclohexane was its low boiling point (80.7°C) which permits its complete removal from aqueous systems by heating (trichloroethylene : Bp = 87.1 °C, toluene : Bp = 110.6 °C). Moreover, it is of acceptable low toxicity and cost (Armbruster, 1988). Trichloroethylene and toluene also produced mainly β-CD product, but they represent greater toxicity hazard ; besides their conversion efficiency were somewhat less than that of cyclohexane. n-Decyl alcohol (decanol), though inhibit CGTase activity by approximately 30 %, would appear to be an attractive approach for α-CD (when high amount of CGTase was used). Unfortunately, its high boiling point (229°C) makes it exceedingly difficult to be removed from aqueous systems by heating. Besides the suitable conversion conditions, properly selected solvent can be used to direct the conversion to provide either α-CD, β-CD, or γ-CD (Armbruster and Jacaway, 1972). After applying

selective conversion conditions, the cyclodextrin can be obtained as the main cyclic compound from a conversion cycle. Complexing agents which are capable of forming complex with β-CD are used in β-CD production. For example, the Chinoin Pharmaceutical Industries patented a process for β-CD production using toluene as the complexing agent (Bender, 1986). Acetone, propanol and ethanol were also reported (Lee and Kim, 1991). For the α-CD production, n-decyl alcohol (or 1-Decanol) was selected as the complexing agent (Flashel, Landert, Renken, 1982), while in γ-CD production, bromobenzene was used (Bender, 1983).

However, by using the two solvents system e.g. cyclohexane-MEK and Nap-MEK as complexing agent, the cyclodextrins yield did not increase. Moreover, the production yield tended to drop comparing with the yield of cyclodextrin production without the use of complexing agent, the same as when only MEK was used. As shown in Table 12, cyclohexane and naphthalein when used alone resulted in an increase in cyclodextrin yield. The decrease in cyclodextrin yield in the presence of cyclohexane-MEK or Nap-MEK should be due to MEK. According to the effect of MEK on CGTase activity (Figure 14), it was found that CGTase activity at the initial time (0′) decreased 0.84 % comparing with the control and during the production time, CGTase activity decreased only 5.89 % comparing with at 0′. However, CGTase activity determined at this point was its dextrinizing activity. CD-forming activity was not checked. MEK might interrupt the formation of cyclodextrins by the enzyme CGTase. Otherwise, it might be a result of the geometrical fit among cyclohexane-CD-MEK and Nap-CD-MEK ternary complexes which may be inappropriate. It has been reported that MEK, possessing a four carbon atom apolar aliphatic chain , showed to be the most appropriate

to form a toluene-CD-MEK ternary complex, with the lowest solubility in water (Seres and Barcza, 1988). The assumed interaction between cyclodextrin and ketone is the hydrogen bonding of carbonyl and secondary alcoholic OH groups. Probably the ketone molecules exist in pre-associated form with toluene and simultaneously with each other by apolar-apolar interaction between their aliphatic ethyl groups. Moreover, the carbonyl group of the second ketone may interact with secondary alcoholic groups of cyclodextrin molecule. This arrangement can induce an appreciable hydrophobic environment in the cyclodextrin cavity through blocking its polar hydrophilic edge. According to this model, the main factor in determining the complex stability is the modified geometrical arrangement. Another example is 1-naphtol (which selectively complexed with β-CD when applied alone) in presence of MEK shifts the cyclization reaction towards γ-CD complex formation (Seres and Barcza, 1988).

Time course of cyclodextrin production was also determined. The result showed that the enzyme CGTase from *Bacillus* sp. A 11 formed mainly β-CD in the initial phase of conversion. Figure 13 showed that the total cyclodextrins rapidly increased in the first 9 hours, then leveled off, while β-CD yield was maximum at around 12 hours. The leveling off could be due to the inactivation of the enzyme or the inhibition of the enzyme by the products or decomposition of cyclodextrins by the enzyme. Since, the activity of CGTase during 24 hours remained stable. The leveling off could thus be due to the other rest reasons.

When performing the time course of cyclodextrin production with the use of complexing agent, the pattern (Figure 15) was similar to that of Figure 12 except that a maximum peak at 18 hours was observed. Figure 16, comparing Figure 15 to Figure 13, showed that the total amount of cyclodextrins with the use of complexing

agent (cyclohexane) was about twice the value obtained when the use of complexing agent is omitted. The increase was due to the fact that the cyclodextrin produced formed an insoluble complex with organic solvents, being precipitated, and the continuous removal of cyclodextrins from the reaction system shifted the equilibrium in favor of cyclodextrin production. Moreover, the result showed that the optimum production time for β-CD production with the use of complexing agent (cyclohexane) was 18 hours and the cyclodextrin yield was mainly from β-CD.

Product separation

Since β-CD is easily separated from the reaction mixture because of its low solubility in water and especially when certain type of complexing agent was used. Such complexing agent permits efficient substrate conversion at high yield and the cyclodextrins accumulated during the conversion was insoluble crystalline complexes of the solvent used. This permits the clean separation of the formed cyclodextrins from non-converted starch by either centrifugation or filtration. In contrast to the non-solvent process where separation of cyclodextrins from non-converted starch is a problem. Direct crystallization gives poor product recovery because the non-converted starch present limits the extent to which the conversion liquor can be evaporated prior to crystallization (Armbruster, 1988). Consequently, several sophisticated recovery procedures have been developed to overcome this problem. Reported procedures include adsorption onto synthetic resin (Horikoshi *et al.*, 1981; Okada, Matsuzawa and Uezima, 1983), fractionation by ultra-filtration (Hashimoto, Hara and Kuwahara, 1986), and separation by use of a dynamic membrane (Hashimoto, Hara and Kuwahara, 1985).

In this study, though we produced cyclodextrins in the presence of complexing agent, the separation of the insoluble cyclodextrin complexes from non-converted starch was not performed. In the small scale preparation, there were some practical problem in the vigorous shaking for the precipitation of cyclodextrin complexes to occur. It was found that the majority of β-CD was still left in the non-converted residual starch (70-80 g % of total cyclodextrins) (data not shown). Therefore, the insoluble cyclodextrin complexes and the residual starch were not separated and they were mixed in the reaction products (Figure 7).

Separation of cyclodextrins from non-cyclic products

Since there were also some linear oligosaccharides contaminated with cyclodextrins in the reaction products (Figure 18), amyloglucosidase (AMG) and β-amylase were used in the attempt to differentiate the linear from the cyclic oligosaccharides. Amyloglucosidase (glucoamylase; Exo-1,4-α-glucosidase) was known to hydrolyze oligosaccharides to glucose while β-amylase acts in the exo-action in hydrolying oligosaccharides to maltose (Bender, 1986). We surprisingly found that AMG not only hydrolyzed linear oligosaccharides, but when used even at 20 units for an hour, was able to destroy γ-CD. The HPLC pattern demonstrated that γ-CD was completely hydrolyzed to glucose (Figure 19). As earlier stated that amyloglucosidase hydrolyzes starch to glucose in the exo-action, thus it should not hydrolyze the cyclic products which have no reducing end. Cyclodextrins were known to resist hydrolysis by exoglucanases, potato phosphorylase (EC 2.4.1.1) (Green and Stump, 1942), sweet potato β-amylase (EC 3.2.1.2) (Thoma and Koshland, 1960), glucoamylase (EC 3.2.1.3) (Numata,

1970) and pullulanase (Saha, Mathupala and Zeikus, 1988), due to the absence of reducing and non-reducing end glucose residues. While endoglucanases e.g. some α-amylases (EC 3.2.1.1) from *Bacillus polymixa*, *Aspergillus oryzae* and *Penicillium africans* have been reported to hydrolyze CDs, but the rate is usually much slower than that of starch (Ben-Gershom and Leibowiz, 1958). Cyclomaltodextrinase (cyclomaltodextrin hydrolase, decycling, CDase) can rapidly hydrolyze CDs but has little or no activity for polysaccharides such as starch and glycogen (Ben-Gershom and Leibowiz, 1958 ; Kitahata *et al.*, 1983). Table 22 lists some cyclodextrin degrading enzymes from microbial sources. Glucoamylase or amyloglucosidase (AMG) from *Flavobacterium* sp. was reported to hydrolyze α-, β- and γ-CD at different degradative rate (Bender, 1981). On the other hands, cyclodextrins are known to resist hydrolysis by the pure glucoamylase of *Aspergillus niger*. However, if a long incubation is performed, γ-CD is hydrolyzed at the faster rate than α- and β-CD (Bender, 1981). Thus,The hydrolysis of γ-CD by amyloglucosidase from *Aspergillus niger* in this work may be as a result of the structural configuration and the stability of γ-CD which is less resistant to hydrolytic enzymes when long incubation time was performed. Otherwise, there may be some contamination of α-amylase or other endo-action hydrolyzing enzymes in the amyloglucosidase preparation. Therefore, besides the type of enzyme and the bacterial source, the purity of enzyme is also a factor in determining the hydrolysis of cyclodextrins.

It was found that all cyclodextrins resisted to β-amylase treatment even when incubated with 40 U for an hour. Maltotetraose (G_4) and maltohexaose (G_6) were completely hydrolyzed to maltose (G_2), while maltopentaose (G_5) was

Table 22 Cyclodextrin degrading enzymes. (Saha and Zeikus, 1992).

Enzyme	Organism	Rate	Reference
Cyclodextrinase	*B. coagulans*	Fast	Kitahata and Tanigushi, 1983
	B. macerans	Fast	Ben-Gershom and Leibowiz, 1958 Depinto and Campbell, 1969 Seres, 1984
	Bacillus sp.	Fast	Kitahata and Tanigushi, 1983
	Bacteroides sp.	-	Antenucci and Palmer, 1984
	B. sphaericus	Fast	Oguma, Kikuchi and Mizusawa, 1990, 1991
	C. thermohydrosulfuricum	Fast	Saha and Zeikus, 1990
Alpha-amylase	*A. oryzae*	Slow	Suetsugu *et al.*, 1974 Abe *et al.*, 1988
	B. subtilis	Very slow	Moseley and Keay, 1970
	F. capsuligenum	Slow	De Mot and Verachtert, 1985b
	Pseudomonas sp.	Fast	Kato *et al.*,1975
	T. pullulans	-	De Mot and Verachtert, 1985a
Glucoamylase	*Flavobacterium* sp.	Fast	Bender, 1981
Amylase	*B. megaterium*	-	David, Gunther and Roper, 1987

hydrolyzed to maltose (G_2) and maltotriose (G_3) (Figure 20). β-Amylase (from sweet potato) was then used in the separation of linear from cyclic oligosaccharides in this study. The result (Figure 20) showed that β-amylase could hydrolyze G_4-G_6 which overlapped with CD peaks and amounted 5-10 g % in the reaction products. Thus, cyclodextrins formed with / without complexing agent were calculated to be 19 and 34 g %, respectively.

Separation of β-CD from cyclodextrin mixtures

In order to prepare β-CD from the production with the use of complexing agent, the separation of cyclodextrins was modified from the flowsheet in Figure 22. Since the solubility of β-CD in water is much lower than α-CD (Table 23), this property facilitates the separation of β-CD from the cyclodextrin mixtures. At 25°C, the solubility of α-CD is 13.0 g/100 ml while the solubility of β-CD is only 1.85 g/100 ml. Thus at 25°C, the α-CD which was present as minor part in the reaction products remained in solution while most of the β-CD crystallized out. In this study, after the reaction products was treated with β-amylase, it was then crystallized. Followed the flowsheet in Figure 7, the result showed that only 1st crystallization can completely separate β-CD from α-CD. Moreover, it was found that crystallization temperature of 4°C yielded twice more β-CD as the 1st crystallization material than the crystallization temperature of 25°C. Since, the solubility depends on the temperature, at 4°C, the solubility of β-CD is lower than at 25°C which makes crystallization better (Table 23). In addition, the result showed that both crystallization temperature of 4°C and 25°C could completely separate

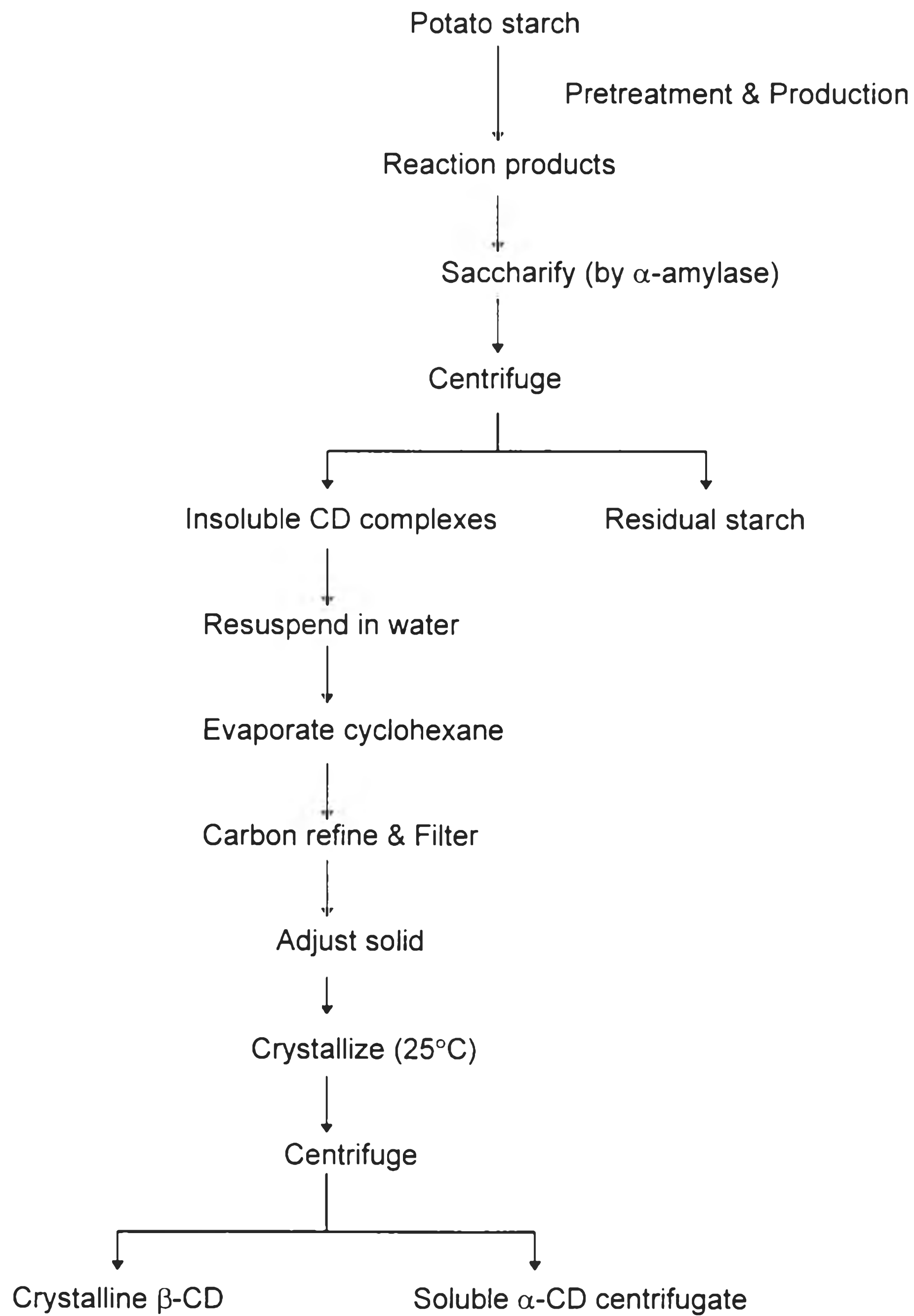

Figure 22 Process flow diagram for β-CD recovery. (Armbruster, 1988)

Table 23 Solubility of cyclodextrins in water. (Nihon Shokuhin Kako, 1987)

Temp. (°C)	Solubility (g/100 ml)		
	α-CD	β-CD	γ-CD
0.5	6.8	0.80	9.1
10.0	7.1	1.15	13.6
15.0	8.6	1.35	18.4
20.0	10.1	1.55	23.2
25.0	13.0	1.85	30.0
30.0	16.0	2.25	38.5
40.0	25.6	3.52	63.5
45.0	32.3	4.45	77.2
50.0	43.5	5.62	93.8
60.0	66.2	9.02	129.2
70.0	87.6	15.30	163.7
80.0	109.3	25.30	198.0

β-CD from α-CD. Even at 25°C, there was no α-CD crystalline contaminated in the 1st crystalline (Table 18). It may be due to low concentration of α-CD in the reaction products and the selectivity of complexing agent which precipitated β-CD as the major product. In this study, the recovery of β-CD from 1st crystallization (at 4°C) was approximately 40 % of that obtained from production prior to crystallization.

In conclusion, by using cyclohexane in the production of cyclodextrins, the crude β-CD obtained was 42 g %. If purer β-CD is preferable, β-amylase treatment was employed to hydrolyze non-cyclic products (34 g %, 80 % recovery) and further purified by crystallization at 4°C. By the 1st crystallization, the β-CD crystals obtained was ≅ 12 g % (28 % recovery, 95 % purity). In comparison with cyclodextrin production from potato starch (30 %, w/v) by *Bacillus macerans* ATCC 8244 CGTase with the use of cyclohexane reported by Armbruster, 1988 (Figure 22), the 1st crystallization yielded β-CD ≅ 22 g % (88 % recovery, 99 % purity). In the Chinoin Pharmaceutical Industries' patent ; using maize starch (28 g %,w/v) as substrate, CGTase from *Bacillus macerans* and toluene as the complexing agent, production yield of 48 g% pure β-CD was reported.

CONCLUSION

1. The CGTase obtained from starch adsorption method, was partially purified to approximately 27-folds with 52 % yield and a specific activity of 2469 Units/mg protein.
2. For cyclodextrin production from rice starch, 10 g % of rice starch was optimally treated with 0.1 % α-amylase, 50°C, 15 minutes. The Dextrose Equivalent (DE) of starch should be in the range of 6-16.
3. The optimum conditions for the production step without the use of complexing agent were pretreated starch incubated with CGTase (25 Units/g starch) for 12 hours. The total cyclodextrin yield was 19 g %, with β-CD as the major product, constituted for 15.6 g %.
4. The optimum conditions for the production step with the use of complexing agent were pretreated starch incubated with CGTase (25 Units/g starch) and cyclohexane (5 % v/v) for 18 hours. The total cyclodextrins yield was 34.2 g %, with β-CD as the major product, constituting for 33.8 g %.
5. With the use of complexing agent (cyclohexane), production yield of cyclodextrins increased about 2 times.
6. Preference production of certain cyclodextrin could be performed by varying the concentration of rice starch or CGTase used. Less than 5 g % starch and more than 200 Units CGTase/g starch mainly yielded α-CD. While 10-30 g % starch and less than 200 Units CGTase/g starch resulted in β-CD as the major product.

7. In the separation of linear from cyclic oligosaccharides, β-amylase (20 Units, 25°C, 1 hr) was successfully used to digest linear oligosaccharides (G_4-G_6) without hydrolyzing the cyclodextrin products.
8. β-CD was separated from total cyclodextrins by crystallization in water at 4°C. Only 1st crystallization can yield pure β-CD.
9. By using cyclohexane in the production of cyclodextrins, the crude β-CD obtained was 42 g %. If purer β-CD is preferable, β-amylase treatment is employed (34 g %, 80 % recovery) and further purified by crystallization at 4°C. By the 1st crystallization, the β-CD crystals obtained was ≅ 12 g % (28 % recovery, 95 % purity).