CHAPTER II

MATERIALS AND METHODS

Equipments

Autoclave : Model HA-30, Hirayama Manufacturing Cooperation, Japan

Bench-top centrifuge : Kokusan Enshinki Co., Ltd., Japan

Refrigerated centrifuge : Model J-21C, Beckman Instrument Inc., USA

High Performance Liquid Chromatography : Model LC-3A Shimadzu, Japan

Incubator : Heraeus, Germany

Shaking water bath : Heto Lab Equipment, Denmark

Water bath : Charles Hearson Co., Ltd., England

Spectrophotometer : Spectronic 20D, Bauch & Lomb, USA

pH meter : PHM 83 Autocal pH meter, Radiometer, Denmark

Vortex : Model K-550-GE, Scientific Industries, USA

Diaflo Ultrafilter : Stirred Ultrafiltration Cell 8050 Amicon W.R. Grace corporation, USA

Filter Holder : Gelman Sciences, Inc., USA

Membrane filters : cellulose nitrate, pore size 0.45 μm, Whatman, Japan

Autopipette : Pipetman, Gilson, France

Chemicals

Acetonitrile (HPLC grade) : J.T. Baker Chemical, USA

Alpha-amylase : (bacterial source) no. 39004 BDH Chemicals, Ltd. England

Amyloglucosidase (Glucoamylase) : 10115 (from *Aspergillus niger*, 120 U/mg), Fluka A.G. Buchs S.G., Switzerland

Beta-amylase : Type I-B from Sweet potato (A 7005), Sigma Chemical Company, USA

Cyclohexane : Farmitalia Carlo Erba, Italy

Soluble starch : Fluka A.G. Buchs S.G., Switzerland

Soluble starch (potato) : Sigma Chemical Company, USA

Standard α -, β -, γ -cyclodextrins : Fluka A.G. Buchs S.G., Switzerland and Sigma Chemical Company , USA

Maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose : Sigma Chemical Company, USA

Toluene : Merck, Germany

Trichloroethylene (TCE) : BDH Laboratory Chemical - Division, England

Other chemicals used were of reagent grade and were purchased from commercial sources. Raw rice starch (three heads elephant brand), corn starch (Maizena) were locally purchased.

Bacteria

Bacillus sp. A 11 was isolated from South-East Asian soil and was a gift from the Division of Microbe Exploration, Fermentation Research Institute, Japan. The strain was reported to have high cyclodextrin-forming ability (Pongsawasdi and Yagisawa, 1987).

Media preparation

1. **Medium I**

Medium I was consisted of beef extract (0.5 %), peptone (1.0 %), NaCl (0.2 %), yeast extract (0.2 %) and soluble starch (Fluka) (1.0 %) and then adjusted to pH 7.2 with 1 N NaOH. For solid medium, 1.5 % agar was added.

2. **Cultivation medium** (Horikoshi's medium)

Medium for enzyme production, slightly modified from Horikoshi (1971); Rutchtorn (1993), contained local grade of rice starch (1.0 %), peptone (0.5 %), yeast extract (0.5 %), K_2HPO_4 (0.1 %), $MgSO_4 \cdot H_2O$ (0.02 %) and Na_2CO_3 (0.75 %). The pH of the medium was 10.1-10.2.

Cultivation of bacteria

1. **Starter inoculum**

Bacillus sp. A 11, after 18 hours inoculation at 37 ^{o}C on solid medium I, was grown into 40 ml of starter medium I in 125 ml Erlenmeyer flask at 37 ^{o}C until A_{420} reached 0.3 - 0.5 or about 3 - 5 hours.

2. **Enzyme production**

Starter *Bacillus* sp. A 11 was transferred into 300 ml Horikoshi's broth in 1000 ml Erlenmeyer flask with 1 % inoculum and cultivated at 37 ^{o}C. Culture was harvested at 72 hours and cells were removed by centrifugation at 3,000 rpm for 30 minutes at 4 ^{o}C. Culture broth with crude CGTase was collected and kept at 4 ^{o}C for purification.

Enzyme assay

For this study, CGTase activity was determined by assay of starch degrading (dextrinizing) activity and assay of CD product by formation of CD - trichloroethylene complex (CD-TCE).

1. Dextrinizing activity assay

Dextrinizing activity of CGTase was measured by the method of Fuwa (1954) with slight modification (Techaiyakul, 1991).

Sample (10-100 μl) was incubated with 0.3 ml starch substrate (0.2 g% soluble starch (potato) in 0.2 M phosphate buffer pH 6.0) at 40°C for 10 minutes. The reaction was stopped with 4 ml of 0.2 N HCl. Then 0.5 ml of iodine reagent (0.02 % I_2 in 0.2 % KI) was added. The mixture was adjusted to a final volume of 10 ml with distilled water and its absorbance at 600 nm was measured. For a control tube, HCl was added before the enzyme sample.

One unit of enzyme was defined as the amount of enzyme which produces 10 % reduction in the intensity of the blue color of the starch-iodine complex per minute under the described conditions.

2. Cyclodextrin-Trichloroethylene (CD-TCE) assay

Cyclodextrin - trichloroethylene (CD-TCE) activity was determined by the method of Nomoto, *et al* (1986) with slight modification (Rojtinnakorn, 1994).

The enzyme sample was diluted in serial double dilution by 0.2 M phosphate buffer, pH 6.0. The reaction mixture, containing 0.5 ml of enzyme sample and 2.5 ml of starch substrate (2.0 %, w/v soluble starch (potato) in 0.2 M phosphate buffer, pH 6.0) was incubated at 4° C for 24 hours. The mixture

was vigorously mixed with 0.5 ml of trichloroethylene (TCE) and left overnight at room temperature in the dark. The activity was expressed in terms of the dilution limit ($1:2^n$), as the highest dilution that can produce observable CD-TCE precipitate lining between upper starch solution layer and lower TCE layer.

Protein determination

Protein concentration was determined by the Coomassie blue standard method according to Bradford (1976), using bovine serum albumin as standard.

One hundred microlitres of sample was mixed with 5 ml of protein reagent and left for 5 minutes before recording the absorbance at 595 nm. One litre of Coomassie blue reagent was the mixture of 100 mg Coomassie blue G-250, 50 ml of 95 % ethanol, 100 ml of 85 % H_3PO_4 and distilled water.

Analysis of cyclodextrins

The sample solutions were analyzed for cyclodextrins by HPLC using Supelco-NH_2 column (0.46 x 25 cm) and RI detector (Rutchtorn, 1992). Prior to injection, the samples were filtered through 0.45 μm membrane filter. The eluent was a mixture of 75 % acetonitrile and 25 % water by volume, and the flow rate was 1 ml min^{-1}. Cyclodextrins detected were analyzed for CD types (α- , β-, γ-CD) by comparing to the retention time of standard cyclodextrins and quantitated from the standard curve of cyclodextrins. Cyclodextrins formed will be expressed as % conversion from starch.

$$\% \text{ Conversion} = \frac{\text{Concentration of cyclodextrins detected (g/l)} \times 100}{\text{Concentration of starch substrate (g/l)}}$$

Dextrose Equivalent (DE) determination

Dextrose Equivalent (DE) is an indirect measure of the degree of hydrolysis of the starch. DE is calculated as % reducing sugar of dry substance.

$$DE = \frac{\text{Reducing sugar (g/l) x 100}}{\text{Dry weight (g/l)}}$$

Reducing sugar determination

Reducing sugar was determined by the method of Nelson Somogyl (Nelson, 1944). Glucose standard (0.1 mg/ml) was prepared. Then 1 ml of sample or standard glucose (0-100 μg/ml) was mixed with 1 ml of Alkaline Copper reagent (prepared as described below). The solution was heated for 10 minutes in a boiling waterbath, then the tubes were cooled in a pan of cold water. Arsenomolybdate reagent (1 ml) was added to each tube, and the solution was left for 15 minutes. Then 5 ml of distilled water was added. After mixing, the absorbance at 520 nm was recorded. Then the quantity of reducing sugar in sample was determined from standard curve of glucose.

Alkaline Copper reagent 1 litre was consisted of Potassium sodium tartrate or Rochelle salt (40 g), $Na_2HPO_4 \cdot 12H_2O$ (71 g), distilled water (700 ml), 1 N NaOH (100 ml), 10 % $CuSO_4 \cdot 5H_2O$ (80 ml). The reagent was left at room temperature for 48 hours before used. The reagent should be stored in a glass-stoppered brown bottle.

Arsenomolybdate reagent 1 litre was consisted of Ammonium molybdate $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (53.2 g), distilled water (900 ml), conc. H_2SO_4 (21 ml), 12 % $Na_2HAsO_4 \cdot 7H_2O$ (50 ml). The reagent was left at room temperature for 48 hours before used. The reagent should be stored in a glass-stoppered brown bottle.

Purification of CGTase

CGTase was partially purified from the culture broth of *Bacillus* sp. A 11 by starch adsorption method of Kato and Horikoshi (1984) with modification (Kuttiarcheewa, 1994) (Figure 5). Five gram percents of corn starch (local grade) was oven dried at 120 °C for 30 minutes and cooled to room temperature. Corn starch was then gradually sprinkled onto stirring crude enzyme broth to make 5 g % concentration. After 3 hours continuous stirring, the starch cake was collected by centrifugation at 5,000 rpm for 30 minutes and washed twice with 10 mM Tris-HCl containing 10 mM $CaCl_2$, pH 8.5 (TB, see appendix A). The adsorbed CGTase was eluted from the starch cake with TB buffer containing 0.2 M maltose (250 ml for starting broth of 1 l), by stirring for 30 minutes and then centrifuged at 5,000 rpm for 30 minutes. The supernatant was further dialyzed in 50 mM Sodium acetate buffer containing 5 mM $CaCl_2$, pH 6.0 (SB, see appendix A). All steps were carried out at 4 ^{o}C.

Cyclodextrin production

The cyclodextrin production process was modified from Nihon Shokuhin Kako Co.'s patent and Chinoin Pharmaceutical Industries 's patent (Bender, 1986) since their protocols were designed for preparation of β-CD as the major product. The process consisted of three main steps which were " Pretreatment of starch " , " Production of cyclodextrins " and " Product separation ". In Nihon Shokuhin Kako Co.'s patent , potato starch was used as the starting material,

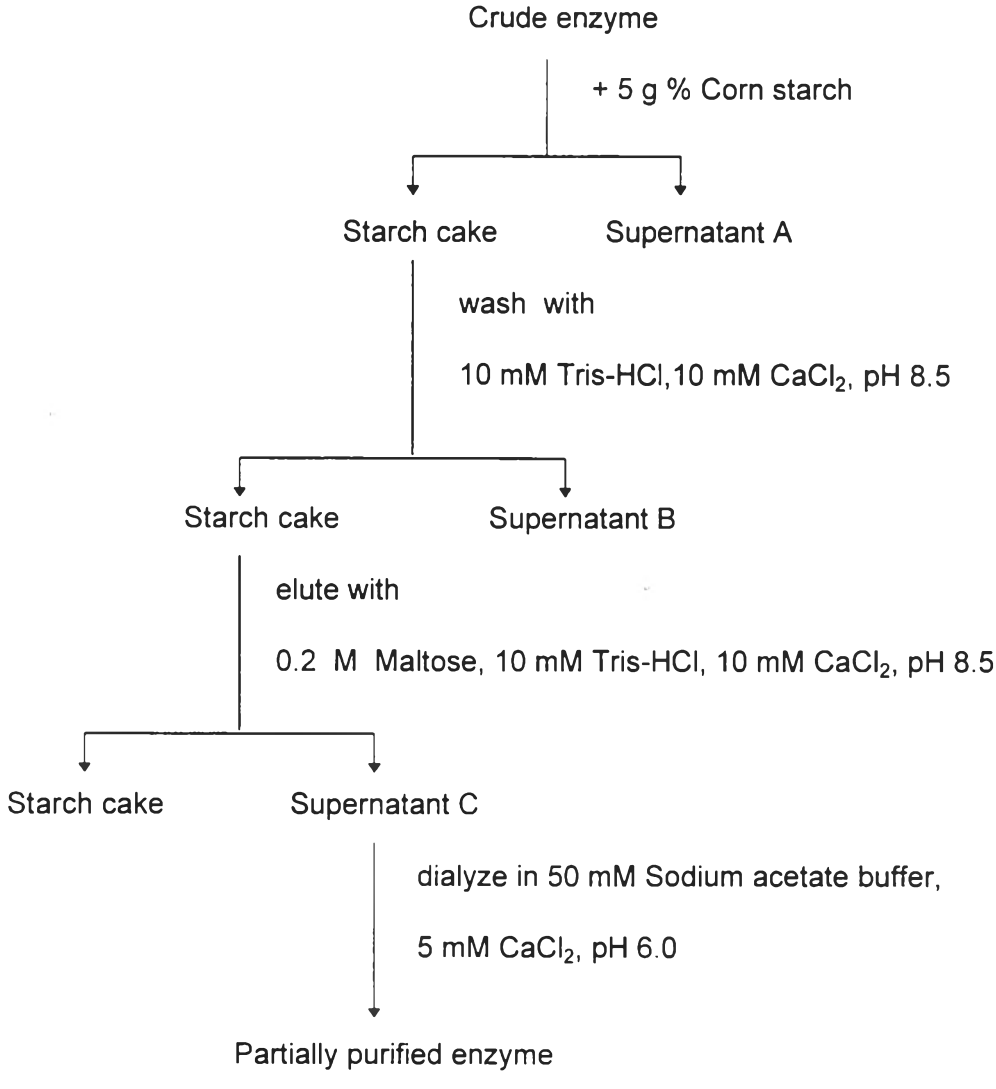

Figure 5 Flowsheet for partial purification of CGTase.

while maize starch was used in Chinoin Pharmaceutical Industries' patent. In this work, the focus was on the development of cyclodextrin production from rice starch. Thus, rice starch was firstly compared to soluble starch (potato) as the starting material for the production of cyclodextrins. Since there was no major practical problem dealing with handling rice starch and the potential of rice starch to be used as starting material exists, detailed steps in pretreatment, production and separation were then optimized. The main theme of the protocol was shown in Figure 6.

1. Optimization of pretreatment step

Pretreatment of starch in the presence of hydrolyzing enzyme, α-amylase was studied. In order to obtain the proper substrate for CGTase, the optimum starch concentration, α-amylase concentration and the incubation time of pretreatment of starch were determined.

1.1 Effect of α-amylase concentration and Dextrose Equivalent (DE) of starch on the formation of cyclodextrins

In the experiment, rice starch (20 %) was pretreated with 0.05-1.0 % α-amylase at 50°C for 1 hour and Dextrose Equivalent (DE) of those pretreated starch was determined as earlier described in this Chapter. Then the dissolved starch was incubated with partial purified CGTase (500 Units/g starch) at 50°C for 17 hours and cyclodextrins were separated from non-cyclic products in the " Product separation step " (Figure 6), and further quantitated by HPLC. Cyclodextrins formed will be expressed as % conversion.

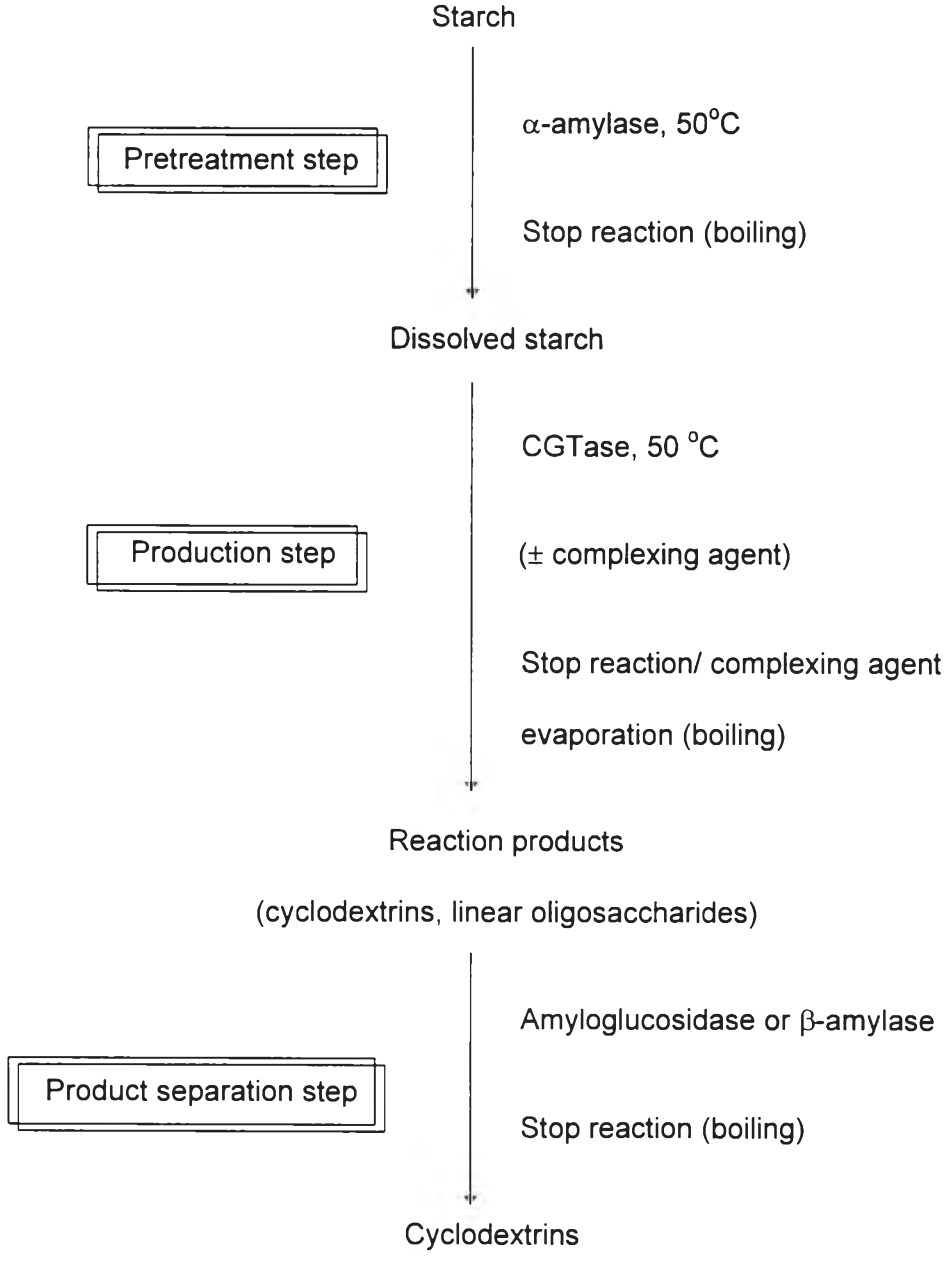

Figure 6 Flowsheet for the production of cyclodextrins.

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

After the optimum concentration of α-amylase was chosen, the pretreated time was varied from 15-60 minutes and Dextrose Equivalent (DE) was determined as earlier described in this Chapter. The dissolved starch was incubated with CGTase under the same conditions as described in 1.1.

1.3 Effect of starch concentration on the formation of cyclodextrins

In the experiment, rice starch varied from 1-30 % (w/v) was pretreated with the optimum conditions in 1.1, 1.2. Then the dissolved starch was incubated with CGTase under the same conditions as described in 1.1.

2. Optimization of production step (without complexing agent)

2.1 Effect of CGTase concentration on the formation of cyclodextrins

Starch concentration and pretreatment of starch were performed according to conditions optimized in Section 1. To test for the influence of CGTase concentration on the yield of cyclodextrins, the treated rice starch was incubated with various amounts of the enzyme (15-1000 Units/g starch) at the same conditions in 1.1.

2.2 Time course of cyclodextrin production

The starch concentration, the pretreatment conditions and the CGTase concentration were fixed at those optimum conditions in Section 1 and 2.1. To determine the time course of cyclodextrin production, the production time was varied from 3-24 hours.

3. Optimization of production steps (with complexing agent)

Cyclodextrin production in the presence of complexing agent was performed following the process in Figure 6. Complexing agent (5 % v/v) was added after 30 minutes of CGTase incubation. The reaction mixture was incubated for 17 hours. Then the complexing agent was evaporated and cyclodextrins were separated from non-cyclic products in the " Product separation step ".

3.1 Effect of complexing agent on CGTase activity

Complexing agents (toluene, trichloroethylene, cyclohexane, methyl-ethyl ketone (MEK), naphthalein (NAP), n-decyl alcohol and ethanol) were checked for their effects on CGTase activity. CGTase was incubated with each complexing agent (5 % ,v/v) at 50°C for 24 hours. Dextrinizing assay was performed at time intervals.

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

Each complexing agent described in 3.1 (5 % v/v or 5 % of 1:1 mixture in case of using two solvent system : NAP-MEK and cyclohexane-MEK) was added to the reaction mixture at the " production step " after 30 minutes of adding CGTase (Figure 5). The treated starch (obtained from optimized conditions in Section 1) and the enzyme CGTase which was varied from 15-1000 Units/g starch were incubated at the same conditions as in 1.1.

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

The treated starch was prepared as the optimum conditions in Section 1, the CGTase concentration was fixed at the optimum conditions in 3.2 and the best complexing agent suitable for cyclodextrin production

chosen from 3.2 was fixed at 5 % v/v. In this experiment, the production time was varied from 3-24 hours.

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

The treated starch was prepared as the optimum conditions in Section 1. The CGTase concentration was fixed at the optimum conditions in 3.2 and the production time was the optimized condition in 3.3. To determine the suitable complexing agent concentration, complexing agent chosen from 3.2 at different concentrations (1-15 % v/v) were added to the production mixture.

4. Product separation

4.1 Separation of cyclodextrins from non-cyclic products

Since there were also linear oligosaccharides detected in the reaction products and some could not be resolved from cyclodextrin peaks in our HPLC system, amyloglucosidase and β-amylase were tested for their hydrolytic actions in the attempt to wipe out the overlapped linear oligosaccharides (non-cyclic products). Standard oligosaccharides, standard cyclodextrins (5 mg/ml) and the reaction products were incubated with 20-40 Units of amyloglucosidase for 1-2 hr at 50°C or with β-amylase at 25°C. After the appropriate enzyme and the suitable treatment conditions were chosen, reaction products from Section 1-3 were treated to get rid of the overlapped linear oligosaccharides by the optimized conditions (Figure 6).

4.2 Separation of β-CD from cyclodextrin mixtures

To separate β-CD which was the major product from cyclodextrin mixtures, crystallization from water was investigated due to the relatively low

solubility in water of β-CD when compared to α- or γ-CD. In the present work, the preparation of β-CD was tried from the production scheme with the use of complexing agent since more β-CD was produced from this protocol (Figure 6). After the reaction products was boiled to evaporate the complexing agent, it was concentrated (2/3 of volume) and treated with the enzyme chosen from 4.1 to hydrolyze non-cyclic products (Figure 7). The sample containing cyclodextrin mixtures was then filtered through a 0.45 μm membrane and crystallized from water. Product crystallization was performed at 25°C and at 4°C for comparison. Crystalline material formed was separated by centrifugation. After that the crude crystalline β-CD was dissolved with hot water and recrystallized by the same method. Products from each step of crystallization were checked for constituents by HPLC.

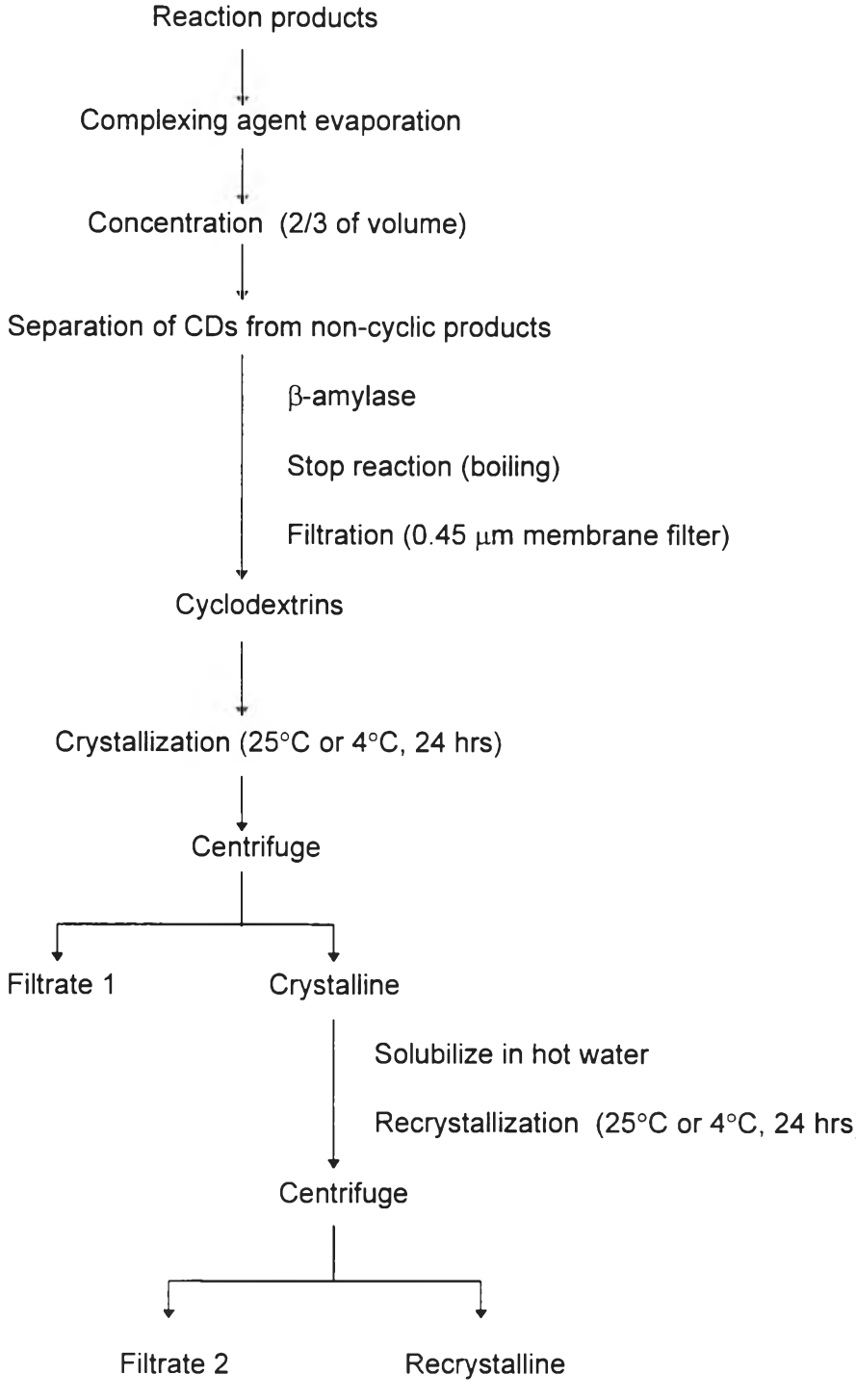

Figure 7 Flowsheet for the separation of β-cyclodextrin.