CHAPTER II

EXPERIMENTS

2.1 Instruments and apparatus

1. LC/MS/MS (Quattomicro, MicromassAPI, UK)
2. HPLC reverse phase column (Asahipak NH2P-50, Shodex, Japan)
3. Nuclear magnetic resonance spectrometer (NMR) (Varian Mercury + 400 NMR Spectrometer, April 2003 version)
4. Hot-plated magnetic stirrer (Corning, USA)
5. Syringe filter (0.45 μm PTFE, Minisart SRP4, Sartorious, Germany)
6. Pipette man (P20, P200, and P5000, Gilson, France)
7. Pipette man (Le100, and Le1000, Nichiryo, Japan)
8. Solvent membrane filters (0.45 μm cellulose, Millipore, USA)
9. Freeze-dryer (Freezone 77520, Benchtop, Labconco, USA)
10. Centrifuge (Centuar 2, Sanyo, UK)
11. Vial-capped 1.5 mL (MCT-150-C, Axygen Scientific, Inc., USA)
12. Filter papers (125 mm ø × 100 circles, Whatman, England.)
13. Rotary evaporator (Buchi rotavapor R-200, Swithzerland)
14. Vacuum oil pump (RV3 rotary vane pump, Edwardsvacuum, England)
15. Electrical food blender 500 watt (Cucina HR 1791/6. Philips, Netherlands)
16. Ultrasonic bath (S30H, 50/60Hz, 275 watts, Elmasonic, England)

2.2 Materials and chemicals

1. Squid pen β-chitin (Ta-Ming Enterprises, Thailand)
2. *N*-acetyl-D-glucosamine (Fluka Chemicals, Ltd., Switzerland)
3. Glccosamine hydrochloride (Fluka Chemicals, Ltd., Switzerland)
4. Glacial acetic acid, analar grade (Merck, Germany)
5. Sodium hydroxide, analar grade (Merck, Germany)
6. Potassium Hydrogen Phthalate (Aldrich, Germany)
7. Sodium hydrogen carbonate (Merck, Germany)
8. Concentrated hydrochloric acid (Merck, Germany)
9. Hydrochloric acid, Analar grade (Merck, Germany)
10. Sodium carbonate, analar grade (Carlo Erba, Italy)
11. Activated charcoal (Fluka Chemicals, Ltd., Switzerland)
12. Absolute ethanol (Merck, Germany)
13. Ethanol commercial grade(Carlo Erba Reagents, France)
14. Potassium Hydrogen pathalate(Fluka Chemicals, Ltd., Switzerland)
15. Duterated dimethyl sulfoxide (Merck, Germany)
16. Duterium oxide (Merck, Germany)

2.3 Shrimp α-chitin (Starting materials)

Shrimp chitin flakes were purchased from Ta-ming Enterprises, Thailand and it is pulverized to fine particle by a 500 watt food blender (Philip HR 1791/6). The size of powder chitin was measured by a sieving shaker at the Metallurgy and Materials Science Research Institute, Chulalongkorn University.

2.4 General procedure for acid hydrolysis of chitin

Chitin (10 g divided into 5 portions) was added portionwise into concentrated hydrochloric acid (50 g, chitin/acid ratio = 1:5 w/w) immersed in a controlled temperature bath for designated period of time. Concentrated hydrochloric acid was pre-warmed to 60 °C and chitin was then gradually added while stirring to prevent excess foaming. For the ultrasonic wave assisted hydrolysis of chitin, the addition of chitin was performed while the acid was sonicated under specific temperature to bring about a total dissolution of chitin (typically take about 20-30 minutes). The chitin solution was allowed for hydrolysis to occur under controlled temperature for designated period of time. The procedure for the reaction monitoring by electrospray ionization mass spectrometry (ESI-MS) and the isolation of the hydrolysis products are described in the subsequent sections.

2.5 Monitoring of hydrolysis of chitin by ESI-MS

2.5.1 Sample preparation

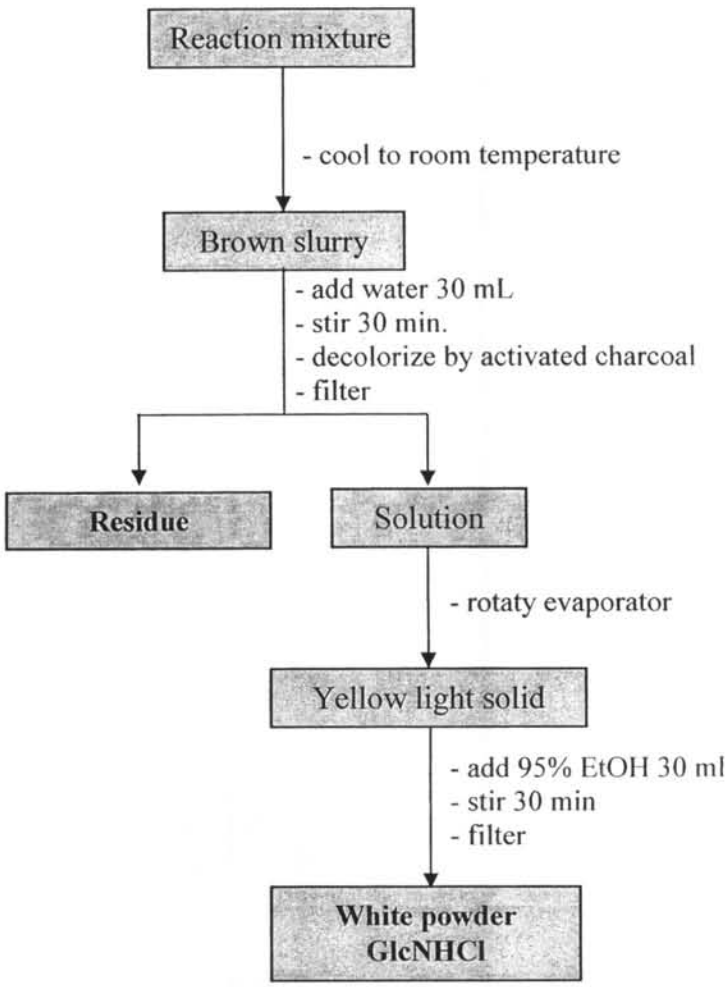
The reaction mixture was stirred at a specified hydrolysis temperature and small amount of hydrolysate (2 mL) was pipetted into absolute ethanol (25 mL) at each time interval. The resulting cloudy mixture was kept in a refrigerator at 4 °C overnight and then centrifuged at 2,000 rpm for 20 minutes. The precipitate was collected by decanting the liquid supernatant off. The precipitate was dissolved in DI-water (4 mL), added with activated charcoal (4 mg) and stirred for 30 minutes. The mixture was filtered though a filter paper (Whatman No. 1) and then a 0.45 μm PTFE filter. The filtrate volume was adjusted to 5 mL by DI-water in a 5 mL volumetric flask and the diluted sample was analyzed by ESI-MS.

2.5.2 ESI-MS analysis

For reaction monitoring, the solution sample (~ 1 ppm, each 1.5 mL) was injected into a mass spectrometer using the optimum injection and ionization parameters; *i.e.* the voltage at capillary, extractor and RF lens were 40 kV, 3 V and 0 V, respectively. The cone voltage was 30 and 35 V for GlcN and GlcNAc, respectively. The source and desolvation temperature were adjusted to 120 and 350 °C, respectively. The desolvation gas flow was 550 L/hr and the cone gas flow was 50 L/hr. Under MS scan mode, all parameters were adjusted to give the highest signals corresponding to GlcN and GlcNAc. GlcN was detected as the signal of $GlcNH^{+}$-H_2O at m/z = 162 and GlcNAc was detected as the signal of $GlcNAcH^{+}$-H_2O at m/z = 204. The relative abundances of these signals were used for plotting against the hydrolysis time.

2.6 Preparation of glucosamine hydrochloride (GlcNHCl)

The reaction apparatus includes a reflux system which comprises a cooled water trap to capture and condense the hydrogen chloride vapor. After the dissolution of all chitin (10 g), the reaction mixture was heated under stirring to a specified hydrolysis temperature and kept at that temperature for a designated period. During the reaction, the viscosity of the solution decreased promptly and a brown color was developed. The reaction slurry was allowed to cool to room temperature. The precipitate containing glucosamine hydrochloride (GlcNHCl) was then dissolved in water (30 mL), stirred for 30 minutes and then decolorized with activated charcoal (0.2 g/100 g initial chitin). The decolorizing solution stirred at room temperature for 30 minutes. The solution was filtered to remove insoluble residue and activated charcoal. The clear filtrate was evaporated to dryness. The light yellow solid was obtained and washed with 95% ethanol (100 mL/100 g initial chitin), stirred for 30 minutes at room temperature and filtered. The white solid was obtained and dried under vacuum to produce the final product, GlcNHCl **(Scheme 2.1)**.

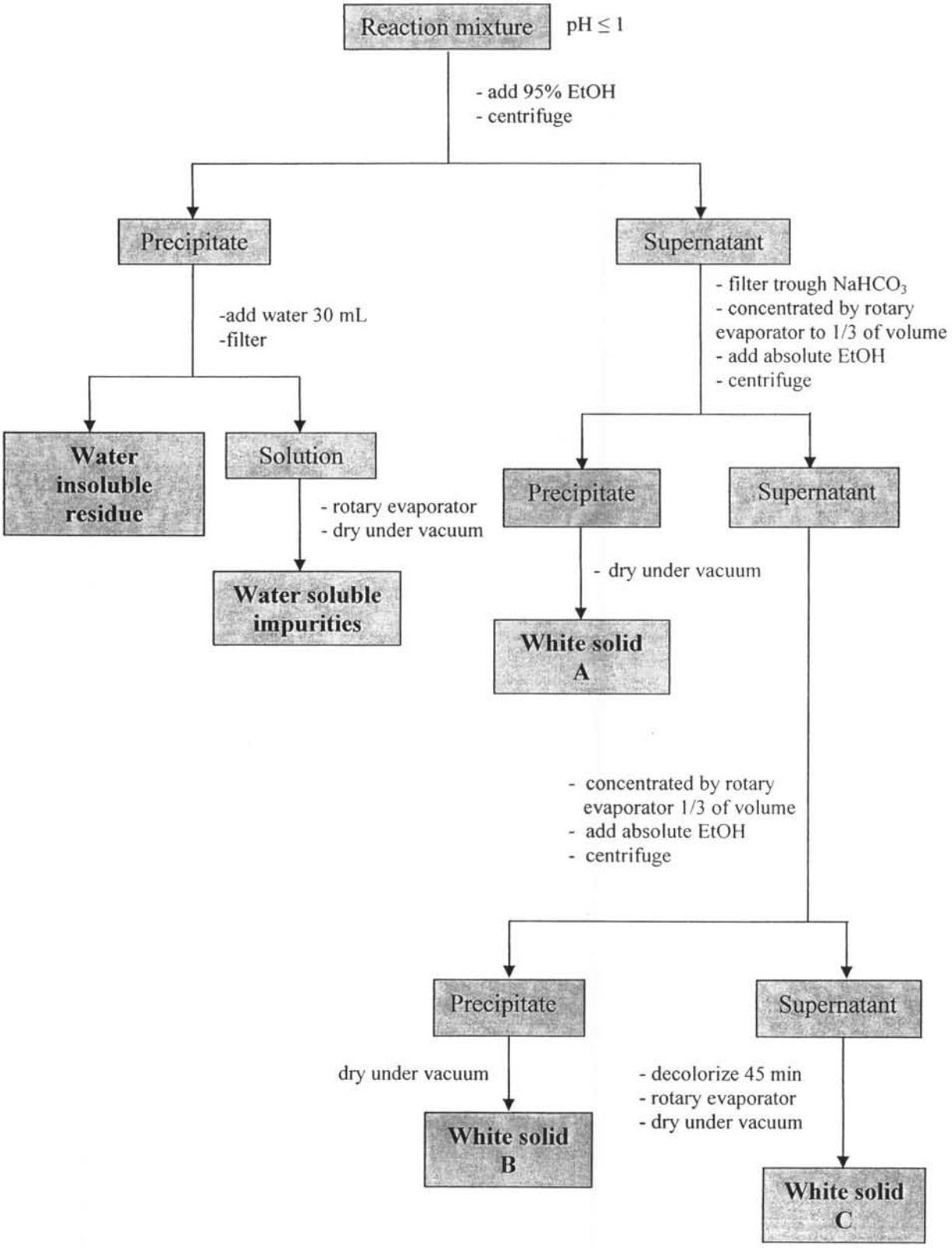


Scheme 2.1 Isolation of GlcNHCl

2.7 Preparation of *N*-acetyl-*D*-glucosamine(GlcNAc)

A chitin solution (10 g chitin/30 mL conc HCl) obtained after sonication was allowed for hydrolysis at controlled temperature. GlcNAc product was isolated according to **Scheme 2.2**. The brown slurry obtained from the hydrolysis was added with 95% ethanol (40 mL) to precipitate a part of impurities. The ethanolic slurry was centrifuged at 2,000 rpm for 20 minutes to remove the remaining chitin and impurities. The obtained precipitate was dispersed in 10 mL of water and filtered to remove the water insoluble residue. The filtrate was then evaporated by rotary evaporator to dryness. The pH of supernatant was adjusted from pH≤1 to neutral by filtering through $NaHCO_3$ powder (40 g). The neutral supernatant was then concentrated to 1/3 of volume by rotating evaporator and dropped into absolute

ethanol (40 mL) while stirring to form a cloudy solution and left in a refrigerator at 4 °C overnight to complete the precipitation. The precipitate was separated by centrifugation at 2,000 rpm for 20 minutes and dried in desiccators under vacuum to afford solid A. The supernatant was concentrated to 1/3 of volume by rotaty evaporator and dropped into cool absolute ethanol to form cloudy solution. The precipitate (solid B) was collected by centrifugation at 2,000 rpm for 20 minutes. The supernatant was decolorized by stirring with activated charcoal for 45 minutes. The mixture was filtered to remove activated charcoal to provide a clear solution. The solution was dried by rotary evaporator and then freeze dried under vacuum to provide white solid (solid C).



Scheme 2.2 Isolation of GlcNAc

2.8 Product analysis

2.8.1 Purity analysis of GlcNHCl by titration

NaOH solution was standardized by potassium hydrogen phthalate (KHP) solution (~0.01 M, 0.5 g/250 mL RO-water) in 50 mL flasks using a few drops of phenolpthalein. The NaOH solution (~0.01 M, 0.2 g/500 mL RO-water) was filled into a buret and slowly added into the KHP solution (10mL) and the titration was repeated two more times. GlcNHCl solution was prepared by dissolving GlcNHCl salt (~0.01 M, 0.1 g/50 mL) in RO-water into 50 mL volumetric flasks and added a few dropped of phenolphthalein. The NaOH solution was slowly added into the GlcNHCl solution and the titration was repeated 2 more times.

2.8.2 Purity analysis of GlcNAc by ESI-Ms

In a sample vial, a solid sample (0.15 g of A, B or C) was dissolved in DI-water (1.5 mL). The solution was filtered trough a 0.45 μm PTFE syringe filter before injecting into a mass spectrometer. Under MS scan mode, all parameters were adjusted to give the highest signals corresponding to GlcNAc. GlcNAc was detected as a signal of $[GlcNAcH^{+}\text{-}H_2O]$ at m/z = 204. The signal abundance was used to calculate the percent purity of GlcNAc against the GlcNAc standard solutions with similar concentration.

2.8.3 ^{1}H NMR of products

In a standard NMR tube, a sample solid (A, B and C) (5mg), was dissolved in dueterated dimethyl sulfoxide ($(CD_3)_2SO$ Merck, 0.5 mL). The solution was filtered trough $NaHCO_3$ (20 mg), dried over 4A molecular sieves overnight and added a drop of deuterium oxide (D_2O) prior to the spectrum acquisition. The spectra of standard GlcNAc and GlcNHCl were acquired from the solutions prepared in a similar manner.