CHAPTER II

EXPERIMENTS

2.1 Instruments and apparatus

1. High performance liquid chromatograph (HPLC): pump (Waters 600E), autosampler (Waters 917), and diode array detector (Waters 996)
2. LC/MS/MS (Quattomicro, MicromassAPI, UK)
3. HPLC reverse phase column (Asahipak NH_2P-50, Shodex, Japan)
4. UV-Visible spectrophotometer (Cary 50 probe, Varian, Australia)
5. Nuclear magnetic resonance spectrometer (NMR) (Varian Mercury + 400 NMR Spectrometer, April 2003 version)
6. pH meter (pHScan3+, Eutech Instruments, Malaysia)
7. Hot-plated magnetic stirrer (Corning, USA)
8. Syringe filter (0.45 μm PTFE, Minisart SRP4, Sartorious, Germany)
9. Pipette man (P20, P200, and P5000, Gilson, France)
10. Pipette man (Le100, and Le1000, Nichiryo, Japan)
11. Solvent membrane filters (0.45 μm cellulose, Millipore, USA)
12. Freeze-dryer (Freezone 77520, Benchtop, Labconco, USA)
13. Centrifuge (Centuar 2, Sanyo, UK)
14. Centrifugal mill (Rector 970, Retsch, Germany)
15. CHON/S analyzer (PE2400 series II, Perkin Elmer, USA)
16. Vial-capped 1.5 mL (MCT-150-C, Axygen Scientific, Inc., USA)
17. Optical microscope (BX60M, Japan)

2.2 Materials and chemicals

1. Squid pen β-chitin (Ta-Ming Enterprises, Thailand)
2. *N*-acetyl-D-glucosamine (Fluka Chemicals, Ltd., Switzerland)
3. *N,N'*-diacetylchitobiose (Seikagaku Corporation Co.Ltd., Japan)
4. Glacial acetic acid, analar grade (Merck, Germany)
5. Sodium chloride, analar grade (Merck, Germany)
6. Sodium hydroxide, analar grade (Merck, Germany)
7. Citric acid, analar grade (Merck, Germany)
8. Sodium hydrogen phosphate (Fluka Chemicals, Ltd., Switzerland)
9. Gel filtration packing material (HW-40S, TOSOH Corporation, Japan)
10. Hydrochloric acid, Analar grade (Merck, Germany)
11. Potassium hexaferrocyanate, (Merck, Germany)
12. Sodium acetate, (Fluka Chemicals, Ltd., Switzerland)
13. Sodium carbonate, analar grade (Carlo Erba, Italy)
14. Potassium hydroxide analar grade (Merck, Germany)
15. Acetronitrile, chromatography grade (Merck, Germany)
16. Acetyl chloride, analar grade (Aldrich, Germany)
17. Sodium deoxycholate (Merck, Germany)
18. Trichloroacetic acid (Merck, Germany)
19. Phosphotungstic acid (Fluka Chemicals, Ltd., Switzerland)
20. Copper sulphate (Fluka Chemicals, Ltd., Switzerland).
21. Activated charcoal (Fluka Chemicals, Ltd., Switzerland)
22. Ethanol (Carlo Erba Reagents, France)

2.3 General procedure

2.3.1 Squid pen chitin 500 μm

The squid pen chitin (β-chitin) was purchased from Ta-ming enterprise Co., Ltd. The chitin was ground to 500 μm by an ultracentrifugal mill (Recter 970) at the Metallurgy and Materials Science Research Institute, Chulalongkorn University. The puffy fibrous chitin was obtained and inspected under optical microscope.

2.3.2 Colloidal chitin

The concentrated hydrochloric acid was gradually added to crab chitin (50 g) in a beaker under vigorous stirring. The added amount of hydrochloric acid (~50 mL) was paused when all chitin solid turned into thick viscous slurry. The viscous slurry was poured as a thin stream into 1 L of a vigorously stirred ice-water mixture to instantly form a fine precipitate. The slurry was kept overnight in a refrigerator at 5 °C. The precipitate was collected by friltration. The white solid was washed successively with DI-water until the filtrate became neutral, and again with copious amount of DI-water (~900 mL).

2.4 Preparation of crude enzyme from fungi (*Aspergillus fumigatus*)

2.4.1 Preparation of potato dextrose broth (PDB) and potato dextrose agar (PDA)

In the enzyme preparation, the potato dextrose broth (PDB) and the potato dextrose agar (PDA) were used for fungi pre-enrichment and cultivation respectively. The PDB was prepared by boiling raw potato cubes ($1\times1\times1$ cm^3, 20 g) in water (1 L) until cooked. After removing of potatoes from the aqueous liquid, pH of the solution was adjusted with hydrochloric acid and sodium hydroxide to 5. The PDB solution was devided into two parts. First part was used for pre-enrichment of lyophilized fungi. Another portion was added with agar powder (2% w/v) and heated. Both solutions, with and without agar, were sterilized under autoclave at 121 °C for 15 minutes. The solution with agar was then poured into culture plates (20 mL/ plate) to give PDA ready for cultivation.

2.4.2 Preparation of colloidal chitin minimum medium (CCMM)

Colloidal chitin minimum medium was used for the production of enzyme from fungi. The colloidal chitin minimum medium contained KH_2PO_4 (1 g), $MgSO_4.7H_2O$ (0.5 g), urea (0.3 g) as a nitrogen source, colloidal chitin (0.5 g dry weight) as a carbon source. The ingredients were mixed in DI-water (1L). The mixture was adjusted to pH 4.5-5.0 by 1 N NaOH or 1 N CH_3COOH and sterilized under autoclave at 121°C for 15 minutes.

2.4.3 Preparation of crude enzyme[59]

Freeze dried fungi *Aspergillus fumigatus* (TISTR 3045) was first cultured in PDB for 5 days or until growth fungi was observed. Then fungal fibrous from PDB were cultured on PDA for 5 days. Fungal inoculum (7 pieces/100mL) on PDA was cultured in CCMM (300 mL) at 30 °C on a rotary shaker at 150 rpm for 9 days. Colloidal chitin (0.5 g dry weight) was added into the CCMM cultivation mixture after 5 and 7 days. Crude enzyme was separated from fungal cells and solid media by centrifugation at 6000 rpm for 15 minutes.

2.5 Crude enzyme from cloned bacteria (*Serratia sp.*)

Crude enzyme from cloned *Serratia sp.* used in this work received from Dr. Rath Pitchyangura Department of Biochemistry, Faculty of Science, Chulalongkorn University. Crude enzyme of *Serratia sp.* showed 4 protein bands posses chitinase activity with molecular weight of 40, 50, 60 and 90 kDa estimated by SDS-PAGE. After chitinase gene (*Chi60* gene) was isolated, the transformant contained a plasmid , PKKChi60, with a 2.8 kb inserted fragment that haboring *Chi60* gene.[68]

2.6 Chitinase activity assaying

2.6.1 Preparation of calibration curve

The stock solution **A** and **B** were prepared by dissolving GlcNAc (11.1 and 10.7 mg) with DI-water 10.0 and 20.0 mL, respectively. The stock solution **A** (5.0179 mM) and stock solution **B** (2.4185 mM) were added with colloidal chitin (50 mg/mL, 50 μL) and the volume was adjusted to 1.5 mL with DI-water to produce standard solutions (No. 1-7 in **Table 2.1**).

Table 2.1 Standard solutions used for creating a GlcNAc calibration line in enzyme assaying

Standard No.	Stock solution	Volume (μL)	amount of GlcNAc (μmole)
1	A	120	0.6021
2	A	100	0.5018
3	B	200	0.4837
4	B	160	0.3870
5	B	120	0.2902
6	B	80	0.1935
7	B	40	0.0967

The control tubes were added only colloidal chitin (50 mg/mL, 50 μL) no GlcNAc standard. The K_3FeCN_6 solution (2 mL of 0.05% w/v in 1 M Na_2CO_3) was pipetted into standard and control tubes, the mixture was imersed in hot water for 15 min. After cooling to room temperature, small particles were removed from the mixture by centrifugation at 2,500 rpm for 15 minutes. The UV-Vis absorbance of standard and control solutions was measured at 420 nm. The standard curve was obtained by plotting ΔA (Absorbance of the control tube - Absorbance of the standard tube) in Y-axis against the amount of GlcNAc (μmole) in X-axis.

2.6.2 Measurement of chitinolytic activity of the enzyme

The chitinolytic activity of the interested enzymes was assayed by measuring the amount of reducing ends, equivalent to GlcNAc, produced in the digestion of colloidal chitin with the enzyme according to Schales method.[69]

The enzyme (50 μL) was pipetted into a clean test tube. The colloidal chitin (50 mg/mL, 50 μL) was added into the tube. Buffer (1 M, 0.15 mL) suitable for each enzyme was added and the reaction volume was adjusted to 1.5 mL by DI-water. The solution was incubated at 37 °C for 30 minutes. After the incubation period, K_3FeCN_6 solution (2 mL of 0.05% w/v in 1 M Na_2CO_3) was added into the test tube. The mixture was heated in boiling water for 15 minutes. After cooling to room temperature, the small particles were removed from the mixture by centrifugation at 2,000 rpm for 15 minutes.

For the control experiment, deactivated enzyme obtained by heating the enzyme solution in boiling water (15 min), was used in place of the active enzyme. The quantity of the reducing sugars was measured by a UV-Vis spectrometer at 420 nm using DI-water as a blank. Both assays and controls were performed in triplicates and the average absorbance (A) was used in the activity determination. The activity unit (U) per volume (mL) of the serum was calculated from the difference of the absorbance (ΔA) between the assay (A_1) and the control (A_0) according to the following equation.

$$\text{Activity (U)} = \mu\text{mole of reducing sugar}/(\text{min}\times\text{mL of serum})$$
$$= (\Delta A/1.195)/(30\times 0.05)$$

One unit (U) of enzyme activity was defined as the amount of enzyme able to produce reducing sugar equivalent to 1 μmole of GlcNAc per min. The factor of 1.195 in the equation was the slope of the calibration line using GlcNAc as a standard reducing sugar (**Figure A1**) and 30 was the incubation time in minutes.

2.7 Product analysis by HPLC

2.7.1 Preparation of calibration curve

Preparation of stock solution I

GlcNAc (2.1 mg) and $(GlcNAc)_2$ (2.1 mg) were dissolved in Milli-Q water (1.0 mL) in each vial. GlcNAc and $(GlcNAc)_2$ solutions (0.7 mL each) were pipetted and mixed together to provide a stock solution which contained 4.75 mM GlcNAc and 2.47 mM $(GlcNAc)_2$.

Preparation of stock solution II

GlcNAc (2.7 mg) and $(GlcNAc)_2$ (2.4 mg) were dissolved in Milli-Q water (1.0 mL) in each vial. GlcNAc and $(GlcNAc)_2$ solutions (0.7 mL each) were pipetted and mixed together to provide a stock solution which contained 6.10 mM GlcNAc and 2.83 mM $(GlcNAc)_2$.

Six standard solutions (A, B, C, D, E, and F) were prepared by dilution of the stock solutions I and II **(Table 2.2)**.

Table 2.2 Preparation of standard solutions of GlcNAc and $(GlcNAc)_2$

Standard solution	(GlcNAc) (mM)	$[(GlcNAc)_2]$ (mM)	Preparation
A	0.095	0.005	Stock solution I (0.03 mL) + H_2O (1.47 mL)
B	0.203	0.094	Stock solution II (0.05 mL) + H_2O (1.45 mL)
C	0.475	0.247	Stock solution I (0.10 mL) + H_2O (0.90 mL)
D	1.020	0.472	Stock solution II (0.20 mL) + H_2O (1.00 mL)
E	1.900	0.988	Stock solution I (0.40 mL) + H_2O (0.60 mL)
F	3.050	1.420	Stock solution II (0.50 mL) + H_2O (0.50 mL)

Each standard (0.300 mL) was mixed with acetonitrile (0.700 mL) and filtered through a 45 μm PTFE membrane filter. The standard solutions (20 μL) were injected into HPLC (pump Waters 600E, Autosampler Waters 917 and diode array detector waters 996) and detected at 210 nm. The mobile phase was acetonitrile:water (70:30) at a flow rate of 1.0 mL/min. The Asahipak NH_2P-50, Shodex (Japan) column was used as the stationary phase. These HPLC analysis conditions were utilized throughout this thesis unless specified otherwise. The calibration curve was obtained by plotting the peak area (mV*sec) as the Y-axis against the concentration of GlcNAc (mM) and $(GlcNAc)_2$ (mM) as the X-axis.

2.7.2 Analysis of products in hydrolysates

After the designated time, each hydrolysate (1 mL) was pipetted into a 2 mL plastic capped vial. The hydrolysate sample was boiled for 15 min and centrifuged at 2,000 rpm for 20 minutes. The supernatant (100 μL) was diluted by milli-Q water (900 μL). The solution (0.300 mL) was pipetted out and mixed with acetronitrile (0.700 mL). The solution was filtered though a 0.45 μm PTFE filter before injecting into the HPLC. The same instrument system and condition as previously described in the preparation of calibration line (**section 3.4.1**) was used in the analysis of the products. The GlcNAc and $(GlcNAc)_2$ were detected at a retention time of 5.6 and 6.5 minutes, respectively. The peak areas were used for the calculation of the amount of GlcNAc and $(GlcNAc)_2$ according to following equation:

$$(\text{GlcNAc})\ (\text{mM}) = \frac{PeakArea}{429.99} \times \text{dilution factor , and}$$

$$[(\text{GlcNAc})_2]\ (\text{mM}) = \frac{PeakArea}{696.49} \times \text{dilution factor}$$

The factors of 429.99 and 696.49 were obtained from the slope of the calibration lines of GlcNAc and $(GlcNAc)_2$ respectively (**Figure A2-A3**).

2.8 Preparation of GlcNAc

2.8.1 Single batch hydrolysis

Squid pen chitin (6 g) was incubated with enzyme from fungi (24 U). The volume was adjusted to 300 mL by using DI-water. Ethanol (15 mL) was added as a preservative agent. The pH of the reaction mixture was adjusted by acetic acid (1 M) to 3. The mixture was incubated at 45 °C for 5 days. After 5 days, the reaction mixture was terminated by immersing the reaction vessel into boiling water for 15 minutes to deactivate the enzyme. The remaining chitin was removed by centrifugation at 2000 rpm for 15 minutes. Crude product was kept in the solid form after freeze dried. The HPLC analysis showed that the crude product contained ~60% (w/w) of GlcNAc.

Crude product (3 g) was redissolved in water (10 mL) and absolute ethanol was added into the sugar solution at various ethanol/water ratios. The cloudy solution was allowed to precipitate at 4 °C in a refrigerator overnight. The precipitate was collected from the solution by centrifugation and dried under vacuum. Both precipitate and supernatant were analyzed by HPLC to determine the recovery and purity of GlcNAc in each fraction.

2.8.2 Fed-batch hydrolysis

The hydrolysis of chitin was performed by fed-batch method starting with 6 g of chitin and 24 U of enzyme in 200 mL of the reaction mixture and another equivalent portion of both reactants was added into the same reaction mixture after 2 days to increase the total concentration of product in the reaction mixture. After 4 days, the reaction mixture was heated in the water bath for 15 min. The resulting hydrolysate (350 mL) was filtered and concentrated to 30 mL using rotating evaporator at the temperature not exceeding 70 °C. The concentrated hydrolysate was added with ethanol at the ethanol/water ratio of 7/1 (v/v). Small amount of precipitate

was obtained and it became insoluble in water. The filtrate was thus evaporated to give a light yellow solid (9 g). The HPLC analysis of this solid showed 91% (w/w) of GlcNAc (71% yield).

In the subsequent experiment, the fed-batch hydrolysis was performed by starting with 10 g of chitin and 40 U of enzyme in 300 mL of the reaction mixture. Chitin and the enzyme were added after 2 and 4 days (5 g/20 U and 10 g/40 U, respectively). After 6 days, the resulting hydrolysate (450 mL) was heated, filtered and concentrated according to the previous experiment. After an addition of ethanol (ethanol/water = 7/1) to this concentrated hydrolysate, the light yellow precipitate (15 g) was obtained. The HPLC analysis of this solid showed 90% (w/w) of GlcNAc (56% yield).

2.8.3 Decolorization of GlcNAc

Since GlcNAc obtained from precipitation method gave light yellow color solid with ~90% purity which is generally unsatisfactory by common standard of chemical reagents. Further improvement in GlcNAc purity was performed by decolorization with activated charcoal. Decolorization of GlcNAc was performed in 3 methods. The GlcNAc solid (~1 g) obtained from the precipitation was redissolved in water (4 mL). Activated charcoal (0.05 g) was added into the solution and stirred at 40 °C for 15 min. The activated charcoal was then removed by filtration using 0.45 μm nylon membrane filter and the filtrate was dried by rotavap. The resulting white solid was dried under vacuum for 24 hr and the purity and recovery of GlcNAc were determined. The improvement of percent recovery of GlcNAc by charcoal washing with either 10% ethanol or water during the filtration step was also investigated.

2.9 Preparation of *N, N'*-diacetylchitobiose [$(GlcNAc)_2$]

2.9.1 Hydrolysis of chitin

Squid pen chitin (9 g) was hydrolyzed by crude enzyme (9 U) from cloned bacteria *Serratia sp.* at pH 6 (adjusted by acetic acid) and 37 °C for 6 days. The reaction mixture was then heated in water bath for 15 min to deactivate the enzyme. The remaining chitin was removed by centrifugation at 2000 rpm for 15 minutes. Crude product was kept in the solid form after freeze dried. The hydrolysis of squid pen chitin at higher enzyme/chitin ratio (5 U/1 g) was also conducted in the similar

procedure. The HPLC analysis showed that the crude product typically contained ~63% $(GlcNAc)_2$.

2.9.2 Precipitation of $(GlcNAc)_2$ from the solution of crude product

The dried crude GlcNAc (3 g) was dissolved in the minimum volume of water (10 mL). Ethanol was added into the sugar solution at various ethanol/water ratios. The precipitate was separated from the solution by centrifugation and dried under vacuum. The dried precipitate was weighed to obtain the percent recovery. The purity of dry precipitate and supernatant were analyzed by HPLC.

2.9.3 Separation of GlcNAc and $(GlcNAc)_2$ by using activated charcoal column[66]

Activated charcoal slurry (60 g) in water (200 mL) was packed in a column (3.7 cm diameter and 10 cm hight). The aqueous solution of the crude product (containing 0.34, 0.44, 0.89 and 1.86 g of GlcNAc/$(GlcNAc)_2$ mixture) was loaded into the column and eluted with a gradient ethanol/water eluent system starting from 0 to 30% ethanol at the flow rate of 2 mL/min. The collected fractions were analyzed by MS/MS in the MRM mode. The chromatogram showing the separation between GlcNAc and $(GlcNAc)_2$ was obtained by plotting the peak area of the signals corresponding to GlcNAc and $(GlcNAc)_2$ as the Y-axis and the retention volume (V_R) as the X-axis. The column resolution (R) was determined according to following equation:

$$R = \delta Z / (W_A/2 + W_B/2)$$

$W_A/2$ and $W_B/2$ are half the peak widths of species A and B respectively. δZ is the distance between (the tops of) both peaks, equivalent to $V_R(B) - V_R(A)$ in this case. The baseline resolution is achieved when $R \geq 1.5$.

2.9.4 Monitoring of the separation between GlcNAc and $(GlcNAc)_2$ by LC/MS/MS

The standard solution of the mixture between GlcNAc and $(GlcNAc)_2$ (1 ppm each) was continuously injected into mass spectrometer. Under MS scan mode, all parameters were adjusted to give the highest signal and the peak width of 0.8-1.0 for both GlcNAc and $(GlcNAc)_2$. At this optimum condition, the mode of analysis was switched to daughter scan using argon gas as a collision media. In the daughter scan mode, the molecular weight of the selected parent ion were 243.880 for $[GlcNAc+Na]^+$ and 446.850 for $[(GlcNAc)_2+Na]^+$. The collision energy was adjusted

to obtain the optimum signals where the height of the parent ion decreased to 10% of the daughter base peak). The optimum parameters (**Table A4-5**) of each sugar were recorded into an MS file in the multiple reaction monitoring (MRM) mode.

A sample of sugar solution (100 μL), from the fractions collected in the elution of the activated charcoal column, was injected into the injection loop (20 μL) of LC/MS/MS while the suitable MS tune page was opened. The optimum parameters created in the MS and inlet files were loaded for each sample. The chromatograms of all daughters selected were obtained. The peak area of the daughter peak with the highest peak area was used for plotting with the retention volume (V_R) eluted from the activated charcoal column to generate the chromatogram showing the separation between GlcNAc and $(GlcNAc)_2$.