CHAPTER II

EXPERIMENTS

1. Instruments and apparatus

1. High performance liquid chromatograph (HPLC): pump (Waters 600E), autosampler (Waters 917), and diode array detector (Waters 996)
2. HPLC reverse phase column (Asahipak NH_2P-50, Shodex, Japan)
3. UV-Visible spectrophotometer (Jenway 600)
4. UV-Visible spectrophotometer (Beckman DU 650)
5. pH meter (pH 3 scan, Eutech Instruments)
6. Hot-plated magnetic stirrer (Coning)
7. Syringe filter (0.45 μm PTFE, Alltech)
8. Pipette man (P20, P200, P1000, and P5000, Gilson)
9. Solvent membrane filters (0.45 μm cellulose, Millipore)
10. Membrane filter (whatmann no. 41)
11. Viscometer (Ubbelohde)
12. Freeze-dryer (Freezone 77520, Benchtop, Labconco)
13. Centrifuge (Centuar 2, Sanyo)
14. Centrifugal mill (Rector 970)
15. Electrical food blender (AB 1088 AIKO, Japan)
16. CHON/S analyser (PE2400 series II, Perkin Elmer)
17. Seemless cellulose tubing (M_w 12000, Wako Chemicals Inc.)
18. Locking dialysis membrane clamps (Membrane Filtration Product Inc.)
19. Vial-capped 1.5 mL (MCT-150-C, Axygen)
20. Gel permeation chromatography (GPC) (PL-GPC110)

2. Chemicals

1. Squid pen β-chitin (Ta-Ming Enterprises, Thailand)
2. Powder β-chitin 3.0 μm (Koyo Chemical, Co.,Ltd., Japan)
3. Chitosan 21 %DA (Koyo Chemical, Co.,Ltd., Japan)
4. Chitosan 16 %DA (Seafresh, Thailand)
5. Chitosan 13 %DA (Ta-Ming Enterprises-Thailand)
6. *N*-acetyl-D-glucosamine (Fluka Chemicals, Ltd., Switzerland)
7. *N,N'*-diacetylchitobiose (Seikagaku Corporation Co.Ltd., Japan)
8. *N,N',N''*-triacetylchitotriose (Seikagaku Corporation Co.Ltd., Japan
9. *N,N',N'',N'''*-tetraacetylchitotetraose (Seikagaku Corporation Co.Ltd., Japan)
10. Chitooligosaccharide mixture (Seikagaku Corporation Co.Ltd., Japan)
11. Skim latex (Pan Asia Biotechnolygy Co., Ltd., Rayong, Thailand)
12. Pectinase P-II *Aspergillus niger*, (Amano Pharmaceutical, Japan)
13. Sodium azide (Riedal-deltaen, Germany)
14. Glacial acetic acid, analar grade (Merck, Germany)
15. Sodium chloride, analar grade (Merck, Germany)
16. Sodium hydroxide, analar grade (Merck, Germany)
17. Citric acid, analar grade (Merck, Germany)
18. Sodium hydrogen phosphate (Fluka Chemicals, Ltd., Switzerland)
19. Gel filtration packing material (HW-40S, TOSOH Corporation, Japan)
20. Hydrochloric acid, Analar grade (Merck, Germany)
21. Potassium hexaferrocyanate, (Merck, Germany)
22. Sodium acetate, (Fluka Chemicals, Ltd., Switzerland)
23. Sodium carbonate, analar grade (Carlo Erba, Italy)
24. Potassium hydroxide analar grade (Merck, Germany)
25. Acetronitrile, chromatography grade (Merck, Germany)
26. Acetyl chloride, analar grade (Aldrich, Germany)
27. Sodium deoxycholate (Merck, Germany)
28. Trichloroacetic acid (Merck, Germany)
29. Phosphotungstic acid (Fluka Chemicals, Ltd., Switzerland)
30. Copper sulphate (Fluka Chemicals, Ltd., Switzerland).

31. Ovalbumin (Aldrich, Germany)
32. Phosphate buffer saline (Merck, Germany)
33. Folin reagent (Aldrich, Germany)

3. General procedure

3.1 Preparation the fresh serum *Hb*

The skim latex from Pan Asia Biotechnology Co., Ltd. (Rayong) was added with the H_2SO_4 (50%, v,v) to adjust the pH to 5.0, the remained rubber particles were settle. The top aqueous layer, serum *Hb*, was filtrated though the membrane filter (whatmann no.41) and the filtrate (serum *Hb*) was stored in a refrigerator at 4 °C for further use.

3.2 Preparation and characterization of substrates

3.2.1 Squid pen chitin 100 μm

The squid pen chitin (β-chitin) was purchased from Ta-ming enterprise Co., Ltd. The chitin was ground by ultracentrifugal mill (Recter 970) at the Metallurgy and Materials Science Research Institute, Chulalongkorn University to give the puffy fibrous chitin with diameter about 50 μm and length about 100 μm. Another batch used finer grinding set up to give fibrous chitin with dimension of 25 μm in diameter and 50 μm in length. Most of our hydrolysis reaction were carried out on fibrous β-chitin substrate with a dimension of 50 μm in diameter and 100 μm in length unless stated otherwise.

3.2.2 Colloidal chitin[86]

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 thick viscous slurry was obtained. The sticky mixture was sonicated for 30-40 min at room temperature. The viscous solution was poured as a thin stream into 1 L of a vigorously stirred ice-water mixture forming instantly a fine precipitate. The slurry was kept overnight in a refrigerator at 5 °C. The precipitate was filtered off, using a G3 sintered-glass funnel. The white solid was washed successively with DI-water and, subsequently, with aqueous sodium hydroxide solution (0.2 M), until the filtrate was neutral, and again with copious amount of DI-water.

3.2.3 Swollen chitin

The squid pen chitin (8.5 g) from Ta-ming enterprise Co., Ltd. was cut into pieces with about 0.5 × 0.5 cm and was put into an electrical food blender (AB 1088 AIKO, Japan). The DI-water (10 mL) was gradually added to soak chitin throughly. The blender was stated and stopped intermittently until all water was absorbed by chitin. The addition of water and grinding process was repeated several times until no more water could be absorbed by chitin.

3.2.4 Determination of %DA of chitin

The chitin substrates were analyzed for percentage of carbon, hydrogen and nitrogen by elemental analyses (PE2400 series II Perkin Elmer). The %DA was calculated from C/N ratio (**Figure A6**).

3.2.5 Chitosan

Chitosan was purchased from three different sources, Koyo Chemical Co.,Ltd., (Japan), Seafresh Co,.Ltd. (Thailand) and Ta-Ming Enterprises (Thailand) (**Table 2.1**). Their molecular weights and %DA were determined in previous work.[87,88]

Table 2.1 The molecular weight and %DA of each chitosans

Source of chitosan	%DA[a]	Molecular weight (M_w)[b]
Koyo chemical (Japan)	21	6,055,920
Seafresh (Thailand)	16	2,840,120
Ta-Ming (Thailand)	13	510,316

[a]Colloidal titration

[b]Gel permeation chromatography

3.3 Protein assaying

3.3.1 Preparation of calibration curve

The stock solution of standard protein was prepared by weighted an ovalbumin, which was the standard protein (100.0 mg) and adjusted volume with phosphate buffer saline (PBS) pH 7.4 to 100.0 mL. The standard protein stock solution was filtered though a low protein binding 0.45μm Millipore filter. The filtrate was calculated for the accurate concentration of protein by measuring the absorbance at 280 nm and using PBS as a blank with UV-Visible spectrophotometer (Beckman DU 650) following the equation

$$A = \varepsilon bc$$

When: A = absorbance, b = 1 cm, ε = 1.064, and c = concentration (mg/mL)

The concentration of the stock solution was varied 5, 10, 20, 40 and 80 μg/mL with NaOH (0.2 M). The standard solution (0.8 mL) was pipetted into the test tube and reagent A (0.3 mL) was added into the solution. The mixture (1.1 mL) was stirred and reagent B (0.1 mL) was added into the mixture. The standard solutions were stirred for 15 min and measured the absorption value at 720 nm for each standard using NaOH (0.2 M) as a blank. The standard curve was obtained by plotting the absorbance in y-axis against the concentration of protein (μg/mL) in x-axis.

3.3.2 Measurement of the protein amount in the serum *Hb*

The serum *Hb* solution (4.0 mL) was pipetted into a clean test tube. The protein in para serum *Hb* was extracted following the ISO/FDIS procedure[89]. The DOC reagent (0.4 mL) was added into the serum *Hb* solution and the mixture was mixed well by vortex. After 10 min, the mixture was added with TCA reagent (0.4 mL) and PTA reagent (0.4 mL). The mixture was stirred again by vortex and waited for 30 min. The mixture was centrifuged at 8,000 rpm for 30 min. The dried precipitate was dissolved in NaOH (0.2M, 0.8mL) and stirred until dissolution was achieved. The amount of protein was determined by following Lawry method[90]. The protein solution was added with reagent A (0.3 mL) and put the reagent B (0.1 mL) and the mixture was stirred and waited for 15 min. The amount of protein was measured by UV-Visible spectrophotometer (Beckman DU 650) at 750 nm. The amount of protein was calculated by comparison with the standard curve of ovabumine protein standard prepared in the previous section (**Figure A5**).

3.4 Chitinase activity assaying

3.4.1 Preparation of calibration curve

The stock solution **A** and **B** were prepared by dissolving GlcNAc (11.1 mg) with DI-water 10.0 and 20.0 mL, respectively. The stock solution **A** (5.0179 mM) and stock solution **B** (2.5089 mM) were diluted to produce standard solutions (**Table 2.2**).

Table 2.2 GlcNAc used and concentration of standard solution for enzyme assaying

Standard No.	Volume (μL) (pipetted stock)	Concentration (μM)	Amount of GlcNAc (μmole)
1	160 (A)	0.5352	0.8029
2	140 (A)	0.4683	0.7025
3	120 (A)	0.4014	0.6021
4	100 (A)	0.3345	0.5018
5	200 (B)	0.3345	0.5018
6	160 (B)	0.2676	0.4014
7	120 (B)	0.2007	0.3011
8	80 (B)	0.1338	0.2007
9	40 (B)	0.0669	0.1004

Each standards were added with colloidal chitin 50μL and made the volume to 1.5 mL with DI-water. The control tube was added only colloidal chitin 50μL and made the volume to 1.5 mL with DI-water. The K_3FeCN_6 solution (K_3FeCN_6 0.05% (w/v) in 1 M Na_2CO_3) 2 mL was pipetted into each standard solutions including the control tube and the mixture was boiled in hot water for 15 min. After cooling at room temperature, small particles were removed from the mixture by centrifugation at 2,500 rpm for 15 mins. The UV-Vis absorption of the standard solutions were 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.

3.4.2 Measurement of chitinolytic activity of the serum *Hb*

The chitinolytic activity of serum *Hb* was assayed quantitatively by measuring the amount of reducing sugars produced in a digestion of colloidal chitin with the serum according to Schales Method.[91]

The serum *Hb* (0.05 mL) was pipetted into a clean test tube. The colloidal chitin (0.05 mL, 50 mg/ml) was added into the tube. Sodium acetate buffer pH 4 (1 M, 0.15 mL) 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, 2 mL of coloring reagent solution (0.5 g K_3FeCN_6/1 L of 0.5 M Na_2CO_3 solution) was added into the test tube. The mixture was heated in boiling water for 15 minutes. After cooling at room temperature, small particles were removed from the mixture by centrifugation at 2,500 rpm for 15 mins.

For the control experiment, the serum was heated in boiling water for 15 min before it was added into the test tube and the same procedure described above was followed. The quantity of the reducing sugars was measured by a UV-Visible spectrophotometer at 420 nm using water as a blank. Both assays and controls were performed in three replicates and the average absorbance (A) was used in the activity calculation. 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.

$$\text{Activity (U)} = \mu\text{mole of reducing sugar/min/mL of serum}$$
$$= (\Delta A/1.0724)/30/0.05$$

The factor of 1.0724 was the slope of the calibration line using GlcNAc as a standard reducing sugar (**section 3.4.1**, **Figure A4**). In this calculation, the incubation time was 30 min.

One unit (U) of enzyme activity was defined as the amount of an enzyme able to produce reducing sugar equivalent to 1 μmole of GlcNAc per min. Specific activity was defined as unit per mg of protein of the enzyme sample.

3.5 Product analysis by HPLC

3.5.1 Preparation of calibration curve

- ***N*-acetyl-D-glucosamine (GlcNAc)**

The samples of exact weight of dried GlcNAc ($C_8H_{15}NO_6$ = 221.21) were dissolved with DI-water (5.00 mL) in two vials to make two stocked solutions, **A** and **B** (**Table 2.3**) The six standard solutions (**C, D, E, F, G, H, I, and J**) were prepared by dilution of **A** and **B**.

Table 2.3 Preparation of GlcNAc standard solutions

Standard solution	Concentration (mg/mL)	Preparation method
A	4.840	**GlcNAc** (24.2 mg) + water 5 mL
B	2.480	**GlcNAc** (12.4 mg) + water 5 mL
C	1.210	**A** (0.500 mL) + water (1.50 mL)
D	0.484	**A** (0.200 mL) + water (1.80 mL)
E	0.242	**A** (0.200 mL) + water (3.80 mL)
F	0.121	**A** (0.100 mL) + water (3.90 mL)
G	0.186	**B** (0.225 mL) + water (2.775 mL)
H	0.062	**B** (0.100 mL) + water (3.90 mL)
I	0.019	**G** (0.200 mL) + water (1.80 mL)
J	0.009	**I** (0.200 mL) + water (1.80 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. The calibration curve was obtained by plotting the concentration of GlcNAc (mM) on the X-axis against the Peak area (mV*sec) on the Y-axis.

- ***N,N'*-diacetylchitobiose ($(GlcNAc)_2$)**

The calibration curve for $(GlcNAc)_2$ ($C_{16}H_{28}N_2O_{11}$ = 424.42) was obtained using the similar procedure described for that for GlcNAc (**Table 2.4**).

Table 2.4 Preparation of $(GlcNAc)_2$ standard solutions.

Standard solution	Concentration (mg/mL)	Preparation method
A	1.600	**$(GlcNAc)_2$** (2.4mg) + water (1.5 mL)
B	1.000	**$(GlcNAc)_2$** (2.1mg) + water (2.1 mL)
C	1.280	**A** (0.4 mL) + water (0.1 mL)
D	0.320	**A** (0.2 mL) + water (0.8 mL)
E	0.600	**B** (0.6 mL) + water (0.4 mL)
F	0.200	**B** (0.2 mL) + water (0.8 mL)
G	0.100	**F** (0.25 mL) + water (0.25 mL)
H	0.050	**F** (0.25 mL) + water (0.75 mL)

- ***N,N',N''*-triacetylchitotriose($(GlcNAc)_3$)**

The calibration curve for $(GlcNAc)_3$ ($C_{24}H_{41}N_3O_{16}$ = 627.63) was obtained using the similar procedure described for that for GlcNAc (**Table 2.5**).

Table 2.5 Preparation of $(GlcNAc)_3$ standard solutions.

Standard solution	Concentration (mg/mL)	Preparation method
A	2.000	**$(GlcNAc)_3$** (3.0 mg)+ water 1.5 mL
B	1.000	**$(GlcNAc)_3$** (1.5 mg) + water 1.5 mL
C	1.600	**A** (0.4 mL) + watre (0.1 mL)
D	1.200	**A** (0.3 mL) + watre (0.2 mL)
E	0.800	**B** (0.4 mL) + water (0.1 mL)
F	0.600	**B** (0.3 mL) + watre (0.2 mL)

Table 2.5 Preparation of $(GlcNAc)_3$ standard solutions (continued).

Standard solution	Concentration (mg/mL)	Preparation method
G	0.400	**B** (0.4 mL) + water (0.6 mL)
H	0.200	**G** (0.25 mL) + watre (0.75 mL)
I	0.050	**G** (0.125 mL) + watre (0.875 mL)

3.5.2 Analysis of products in hydrolysates

After the designated time, each hydrolysate (100 μL) was pipetted into a 2 mL plastic capped vial and diluted with DI-water (900 μL) (dilution factor = 10) The mixture was boiled for 15 min and centrifuged at 2,000 rpm for 20 minutes. The supernatant (0.3 mL) was pipetted out and mixed with acetronitrile (0.7 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 3.5.1**) was used in the analysis of the products. The GlcNAc, $(GlcNAc)_2$ and $(GlcNAc)_3$ were detected at a retention time of 5.6, 6.5, and 7.9 min respectively. The peak areas were used to calculate the amount of the GlcNAc, $(GlcNAc)_2$ and $(GlcNAc)_3$ according to following equation:

$$[\text{GlcNAc}]\ (\text{mM}) = \frac{\text{PeakArea}}{353.29} \times \text{dilution factor , and}$$

$$[(\text{GlcNAc})_2]\ (\text{mM}) = \frac{\text{PeakArea}}{495.54} \times \text{dilution factor}$$

$$[(\text{GlcNAc})_3]\ (\text{mM}) = \frac{\text{PeakArea}}{834.32} \times \text{dilution factor}$$

The factors of 353.29, 495.54, and 834.32 were obtained from the slope of the calibration lines of GlcNAc, $(GlcNAc)_2$ and $(GlcNAc)_3$, respectively (**Figure A1-A3**).

3.6 Study for the optimum condition for hydrolysis of chitin with the serum *Hb*

3.6.1 The effect of enzyme:chitin ratio

The ratio between the serum *Hb* and fibrous β-chitin was varied in this experiment. The fibrous β-chitin (100 mg) was weighted into a screw-capped vial. The sodium azide solution (1%, 0.5 mL) and acetate buffer pH 4 (0.5 M, 0.5 mL) were added. The serum *Hb* (10.6 mU) was added and made the reaction volume to 5.0 mL by DI-water. The hydrolysis was carried out at 37 °C under continuous stirring of magnetic stirrer. Various amounts of the serum *Hb* were used (**Table 2.6**). The reactions were monitored on the 1st, 2nd, 4th, 6th and 8th days by HPLC.

Table2.6 The amounts of reagents used in reactions in the study for optimum enzyme/chitin ratio.

Code	Chitin (mg)	Serum *Hb* (mU)	**Ratio serum:Chitin (mU/mg)**	NaOAc 0.5M (mL)	1% NaN_3 (mL)	DI-water (mL)
RSC0.11	100	10.6	**0.11**	0.5	0.5	3.9
RSC0.54	100	53.7	**0.54**	0.5	0.5	3.5
RSC1.07	100	107.4	**1.07**	0.5	0.5	3.0
RSC1.61	100	161.1	**1.61**	0.5	0.5	2.5
RSC2.15	100	214.8	**2.15**	0.5	0.5	2.0

3.6.2 The effect of concentration of chitin

In this experiment, the concentration of chitin was varied from 5 mg/mL to 70 mg/mL, while the amount of the serum *Hb* was also varied to keep the ratio between the serum *Hb* and chitin constant at the optimum ratio obtained in the previous experiment. The appropriate amount of chitin and the serum *Hb* were placed in a screw-capped vial. Sodium acetate buffer pH 4 (1 M, 0.5 mL) and sodium azide solution (1%, 0.5 mL) were added into the hydrolysis reaction. The volume of the reaction was made to 5.0mL by DI-water (**Table 2.7**). The hydrolysis was carried out at 37 °C under continuous stirring of a magnetic stirrer. The reactions were monitored on the 1st, 2nd, 4th, 6th and 8th days by HPLC.

Table 2.7 The amounts of reagents used in the reactions in the study for optimum concentration of chitin

Code	**Chitin (mg)**	Serum *Hb* (mU)	NaOAc 0.5M (mL)	1% NaN_3 (mL)	DI-water (mL)
CC5	**25**	5.3	0.5	0.5	3.95
CC10	**50**	10.7	0.5	0.5	3.90
CC20	**100**	21.3	0.5	0.5	3.80
CC30	**150**	32.0	0.5	0.5	3.70
CC40	**200**	42.7	0.5	0.5	3.60
CC50	**250**	53.3	0.5	0.5	3.50
CC60	**300**	64.8	0.5	0.5	3.40
CC70	**350**	74.7	0.5	0.5	3.30

3.6.3 the effect of pH of the reaction solution

In this experiment, the pH of the reaction was varied from 2.0 - 5.5 by using citrate phosphate (McIlvan) and acetate buffer. The fibrous β-chitin (300 mg) was placed in a screw-capped vial. The mixture of serum *Hb* (64.8 mU), NaN_3 (1%, 0.5mL) and the appropriate buffer solution was added and made the solution to 5.0 mL by DI-water (**Table 2.8**). The hydrolysis was carried out at 37 °C under continuous stirring of a magnetic stirrer. The reactions were monitored on the 1st, 2nd, 4th, 6th and 8th days by HPLC.

Table 2.8 The amounts of reagents used in reaction in the study for optimum pH.

Code	Chitin mg	Serum *Hb* (mU)	Buffer			1% NaN_3 (mL)	DI-water (mL)
			pH	Types (M)	Volume (mL)		
CS-C2.0	300	64.8	**2.0**	McIlVan (0.5M)	1.0	0.5	3.4
CS-M2.5	300	64.8	**2.5**	McIlVan (0.5M)	1.0	0.5	2.9
CS-M3.0	300	64.8	**3.0**	McIlVan (0.5M)	1.0	0.5	2.9
CS-M3.5	300	64.8	**3.5**	McIlVan (0.5M)	1.0	0.5	2.9

Table 2.8 The amounts of reagents used in reaction in the study for optimum pH (continued).

Code	Chitin mg	Serum *Hb* (mU)	Buffer			1% NaN_3 (mL)	DI-water (mL)
			pH	Types (M)	Volume (mL)		
CS-M4.0	300	64.8	**4.0**	McIIVan (0.5M)	1.0	0.5	2.9
CS-M4.5	300	64.8	**4.5**	McIIVan (0.5M)	1.0	0.5	2.9
CS-A4.0	300	64.8	**4.0**	Acetate (1M)	0.5	0.5	3.4
CS-A4.5	300	64.8	**4.5**	Acetate (1M)	0.5	0.5	3.4
CS-A5.0	300	64.8	**5.0**	Acetate (1M)	0.5	0.5	3.4
CS-A5.5	300	64.8	**5.5**	Acetate (1M)	0.5	0.5	3.4

3.6.4 The effect of types of buffer

The hydrolysis reactions of fibrous β-chitin with serum *Hb* were carried out in various types of buffer pH 4.0 (sodium acetate, potassium acetate, citrate phosphate (McIIvan), sodium citrate and potassium hydrogen phthalate (KHP)). Chitin (300 mg) was put into a screw-capped vial. The mixture of serum *Hb* (64.8 mU), NaN_3 (1%, 0.5mL) and to appropriate buffer pH 4.0 was added and made the solution to 5.0 mL by DI-water. The hydrolysis was incubated at 37 °C under continuous stirring of magnetic stirrer (**Table 2.9**). The reactions were monitored on the 2nd and 4th days by HPLC.

Table 2.9 The amounts of reagents used in the reaction in the study for suitable buffers.

Code	Chitin mg	Serum *Hb* (mU)	Buffer		1% NaN_3 (mL)	DI-water (mL)
			Types (M)	Volume (mL)		
TB-AN4.0	300	64.8	**Sodium Acetate (1M)**	0.5	0.5	3.4
TB-AK4.0	300	64.8	**Potassium Acetate (1M)**	0.5	0.5	3.4
TB-M4.0	300	64.8	**McIIVan (0.5M)**	1.0	0.5	2.9

Table 2.9 The amounts of reagents used in the reaction in the study for suitable buffers (continued).

Code	Chitin mg	Serum *Hb* (mU)	Buffer		1% NaN_3 (mL)	DI-water (mL)
			Types (M)	Volume (mL)		
TB-C4.0	300	64.8	**Citrate (1M)**	0.5	0.5	3.4
TB-K4.0	300	64.8	**KHP (0.5M)**	1.0	0.5	2.9

3.6.5 The effect of temperature

The hydrolysis reactions of fibrous β-chitin with serum *Hb* were incubated at various temperatures (30, 37, 45 and 55°C). The chitin (300 mg) was placed in a screw-capped vial. The mixture of serum *Hb* (64.8 mU), NaN_3 (1%, 0.5mL) and acetate buffer pH 4.0 (1M, 0.5mL) was mixed and made the solution to 5.0 mL by DI-water (**Table 2.10**). The reaction mixtures were incubated under continuous stirring of magnetic stirrer. The supernatants were monitored on the 1st, 2nd, 4th, 6th and 8th days by HPLC.

Table 2.10 The amounts of reagents used in the reactions in the study for optimum temperature.

Code	Chitin mg	Serum *Hb* (mU)	Acetate Buffer (1M, mL)	**Temperature (°C)**	1% NaN_3 (mL)	DI-water (mL)
T-30	300	64.8	0.5	**30**	0.5	3.4
T-37	300	64.8	0.5	**37**	0.5	3.4
T-45	300	64.8	0.5	**45**	0.5	3.4
T-55	300	64.8	0.5	**55**	0.5	3.4

3.6.6 The effect of concentration of buffer

The hydrolysis reactions of fibrous β-chitin with serum *Hb* were carried out in various concentration of buffer pH4 (sodium acetate buffer). The chitin (300 mg) was placed in a screw-cap vial. The mixture of serum *Hb* (64.8 mU), NaN_3 (1%, 0.5 mL) and buffer pH 4.0 with appropriate concentration was added and made the solution to 5.0 mL by DI-water (**Table 2.11**). The hydrolysis was incubated at 45 °C under continuous

stirring of magnetic stirrer. The reactions were monitored on the 1st, 2nd, 4th, 6th and 8th days by HPLC.

Table 2.11 The amounts of reagents used in the reaction in the study for suitable concentration of buffers.

Code	Chitin (mg)	Serum *Hb* (mU)	Acetate Buffer (1M)		1% NaN_3 (mL)	DI-water (mL)
			Volume (mL)	**Concentration (M)**		
CB-0.0	300	64.8	0.0	**0.0**	0.5	3.9
CB-0.05	300	64.8	0.25	**0.05**	0.5	3.65
CB-0.1	300	64.8	0.5	**0.1**	0.5	3.4
CB-0.2	300	64.8	1.0	**0.2**	0.5	2.9
CB-0.4	300	64.8	2.0	**0.4**	0.5	1.9

3.6.7 Substrate dependence

The substrate dependence was studied in this experiment. The reactions were incubated with substrates obtained from different preparation methods (see preparation at **section 3.2.1**). The mixture of chitin, serum *Hb*, NaN_3 (1%, 0.5mL) and buffer pH 4.0 (1 M, 0.5 mL) was added and made the solution to 5.0 mL by DI-water (**Table 2.12**). The hydrolysis was incubated at 45 °C under continuous stirring of magnetic stirrer. The reactions were also monitored on the 2nd, 4th, 6th and 8th days by HPLC.

Table 2.12 The types of substrate and reagents used in the reaction in the study for substrate dependence.

Chitin		Serum *Hb*	Acetate Buffer	1% NaN_3	DI-water
Type	(mg/mL)	(mL, mU)	(1M, mL)	(mL)	(mL)
Squid pen chitin 100 × 50 μm	60	64.8	0.5	0.5	3.16

Table 2.12 The types of substrate and reagents used in the reaction in the study for substrate dependence (continued).

Chitin		Serum *Hb*	Acetate Buffer	1% NaN_3	DI-water
Type	(mg/mL)	(mL, mU)	(1M, mL)	(mL)	(mL)
Squid pen chitin 50 × 25 μm	60	64.8	0.5	0.5	3.16
Powder chitin 3.0 μm	60	64.8	0.5	0.5	3.16
Squid pen chitin 100 × 50 μm	20	21.6	0.5	0.5	3.72
Colloidal chitin	20	21.6	0.5	0.5	3.72

3.6.8 Hydrolysis with increasing enzyme/chitin ratio

The enzyme/chitin ratio was increased in this investigation. The fibrous β-chitin (300 mg) was weighted into a screw-capped vial. NaN_3 (1%, 0.5 mL) and acetate buffer pH 4.0 (0.5 M, 0.5 mL) were added. The appropriate amount of serum *Hb* was added and made the reaction volume to 5.0 mL by DI-water (**Table 2.13**). The hydrolysis was carried out at 45 °C under continuous stirring of a magnetic stirrer. The reaction were incubated at 45 °C and monitored on 2nd, 4th, 6th, and 8th days by HPLC.

Table 2.13 The amounts of reagents used in reactions in the study for hydrolysis with increasing enzyme/chitin ratio.

Code	Chitin (mg)	Serum *Hb* (mU)	**Ratio serum:Chitin (mU/mg)**	NaOAc 0.5M (mL)	1% NaN_3 (mL)	DI-water (mL)
CS1F	300	64.8	**0.22**	0.5	0.5	3.16
CS2F	300	129.6	**0.43**	0.5	0.5	2.32
CS3F	300	194.4	**0.64**	0.5	0.5	1.48
CS4F	300	259.2	**0.86**	0.5	0.5	0.64

3.6.9 Product inhibition by GlcNAc

The product inhibition was investigated by carrying out the hydrolytic reactions in the presence of GlcNAc intentionally added in various concentrations at the beginning of the reactions. The GlcNAc (0, 10, 20, 30, 40, and 50 mg) were weighted into six screw-capped vial containing fibrous β-chitin (300 mg). NaN_3 (1%, 0.5 mL) and acetate buffer pH 4 (1 M, 0.5 mL) also added into the reaction and made the volume to 5.0 mL (**Figure 2.14**). The yield of products, GlcNAc and $(GlcNAc)_2$ were monitored at 2nd and 6th day by HPLC.

Table 2.14 The amounts of reagents used in the reaction in the study for GlcNAc inhitbition.

Code	Chitin (mg)	**GlcNAc (mg)**	Serum *Hb* (mU)	1% NaN_3 (mL)	Acetate buffer (mL)	DI-water (mL)
PI0	300	**0**	64.8	0.5	0.5	5.0
PI10	300	**10**	64.8	0.5	0.5	5.0
PI20	300	**20**	64.8	0.5	0.5	5.0
PI30	300	**30**	64.8	0.5	0.5	5.0
PI40	300	**40**	64.8	0.5	0.5	5.0
PI50	300	**50**	64.8	0.5	0.5	5.0

3.7 The mechanistic pathways of chitinolysis

3.7.1 Hydrolysis of $(GlcNAc)_2$, $(GlcNAc)_3$, and $(GlcNac)_4$

Dried $(GlcNAc)_2$ (1.5 mg, 3.59 μmole) was weighted in to a screw-capped vial 2.0 mL. The serum *Hb* (3.8 mU), NaN_3 (1%, 0.1 mL) and acetate buffer pH 4 (1 M, 0.1 mL) were added and the total volume of the reaction were adjusted by DI-water to 1.0 mL (**Table 2.15**). The reaction were stirred continually by a magnetic stirrer and incubated at 45 °C. The reactions were monitored at 1 hr, 3 hrs, 5 hrs and 24 hrs by HPLC.

That of the $(GlcNAc)_3$ and $(GlcNAc)_4$ were also hydrolyzed with the same procedure of hydrolysis of $(GlcNAc)_2$ described above.

Table 2.15 The amounts of reagents used in the reaction in the study for hydrolysis of $(GlcNAc)_2$, $(GlcNAc)_3$, and $(GlcNAc)_4$

Code	$(GlcNAc)_2$ (mg, μmole)	Serum *Hb* (mU)	acetate (1M) (mL)	1% NaN_3 (mL)	DI-water (mL)
Hy-dimer	1.5, 3.59	3.8	0.1	0.1	0.75
Hy-trimer	2.3, 3.59	3.8	0.1	0.1	0.75
Hy-tetramer	3.0, 3.59	3.8	0.1	0.1	0.75

3.8 Preparative scale preparation of *N*-acetyl-D-glucosamine and *N,N'*-diacetyl-chitobiose by using dialysis tubing

In **System A1**, the fibrous β-chitin (3.2 g) and enzyme from serum *Hb* (0.79 U) were placed separately to two dialysis bags (molecular weight cutoff 12,000). The sodium acetate buffer pH 4.0 (1 M, 3.0 mL) and NaN_3 (1%, 3.0 mL) were added into each dialysis bags and the total volume was made to 30.0 mL with DI-water. The dialysis bags were sealed and placed in a beaker containing acetate buffer pH 4.0 (0.1 M) and NaN_3 (1000 ppm) to make the total volume to 750 mL. The outer solution was stirred with magnetic bar tied to the dialysis bags that caused rotating motion of the bags inside the beaker (**Figure 2.1**).

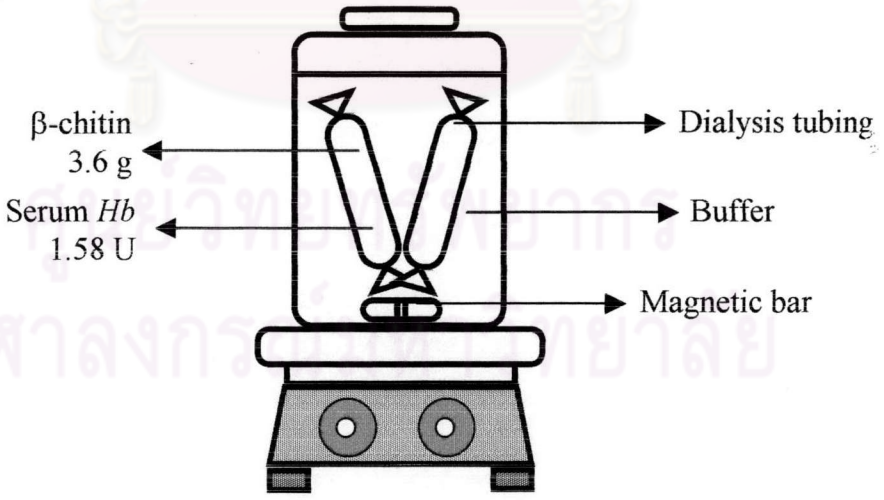

Figure 2.1 Schematic representation of **System A**

The outer solution (500 mL) was refreshed after 11 days when the concentration of product in the outer solution seemed constant. Another portion of the serum *Hb* (0.79 U) was also added inside the bags. The reaction was continued for 10 more days. The combined outer solution was centrifuged at 3000 rpm for 20 min. The supernatant was dried by freeze-dryer to give a crude solid (4.75 g) of GlcNAc (2.6 %, w/w) and $(GlcNAc)_2$ (12.5%, w/w). The isolation of $(GlcNAc)_2$ from this mixture was described in the next section.

In **System A2**, swollen squid chitin (3.2 g, see preparation at **section 3.2.3**) was hydrolyzed in place of fibrous β-chitin with the enzyme from the serum *Hb* (1.58 U) in two dialysis bags (**Figure 2.1**). The reaction was incubated under the same condition as described for **System A1** excepted for that the initial amount of enzyme was double and no second addition of the enzyme.

In **System B**, the hydrolysis reaction was carried out in three dialysis bags. Each bag containing enzyme serum *Hb* (1.06 U) and fibrous β-chitin (2.4 g). The mixture in dialysis tubing was agitated by air bubbling (**Figure 2.2**). The other conditions were similar to those described for **System A2**.

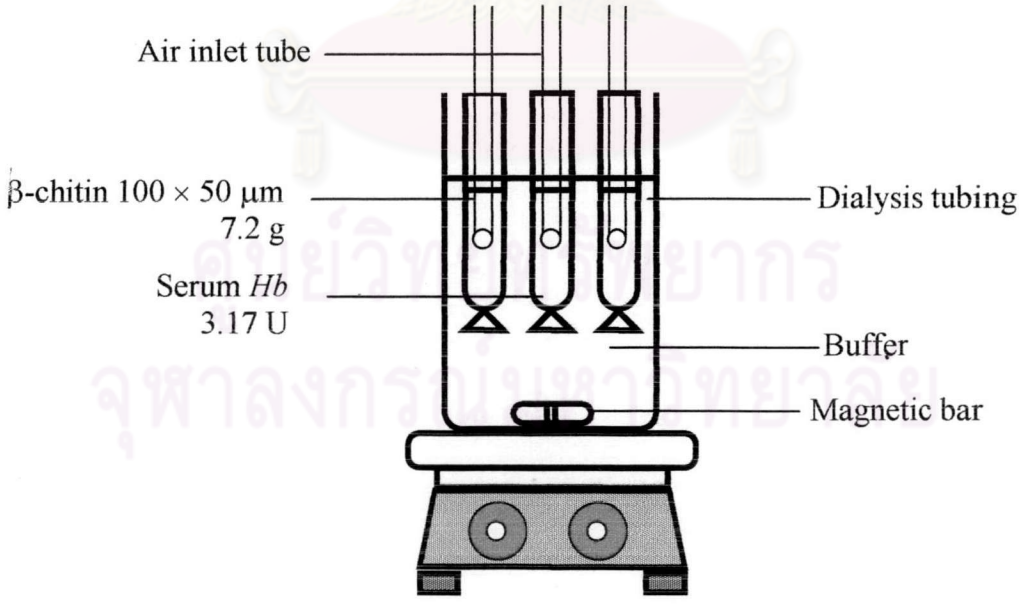

Figure 2.2 Schematic representation of **System B**

In **System C**, the fibrous β-chitin (14.4 g) was incubated with enzyme serum *Hb* (6.34 U) in six dialysis bags without any agitation inside the bags. Each bag contained fibrous β-chitin (2.4 g) and serum *Hb* (1.06 U) The reaction was carried out at 45 °C with continuous stirring under condition similar to those described for **System B** (**Figure 2.3**).

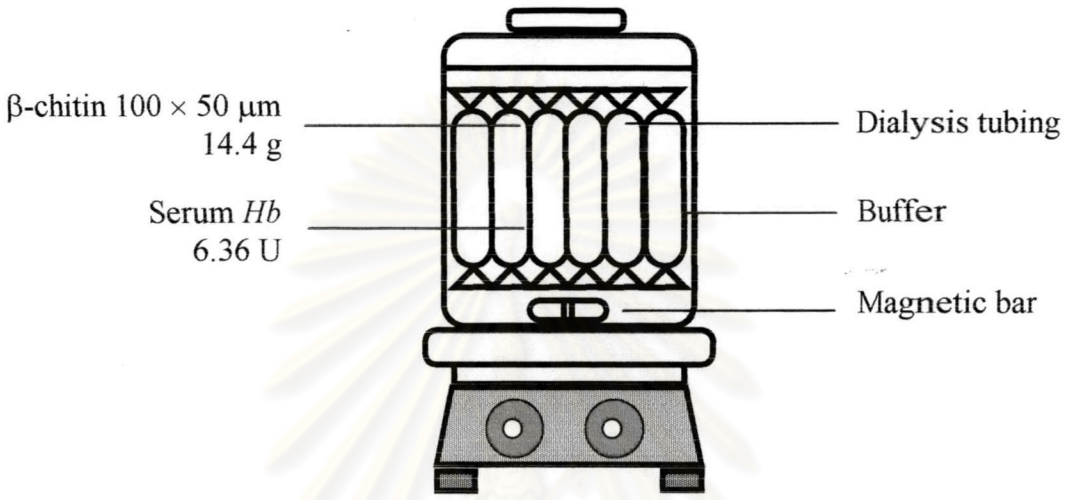

Figure 2.3 Schematic representation of **System C**

3.9 Purification of *N*-acetyl-D-glucosamine and *N,N'*-diacetylchitobiose

3.9.1 Production the *N*-acetyl-D-glucosamine in one-step

Instead of attempting to separate GlcNAc from $(GlcNAc)_2$, the enzyme mixing technique was used to convert all $(GlcNAc)_2$ to GlcNAc.[51] The crude enzyme pectinase from *Aspergillus niger* (Amano, Japan) was used for mixing with serum *Hb* as it was known to possess high β-*N*-acetylhexosaminidase.[51]

The activity of β-*N*-acetylhexosaminidase, chitobiase, of pectinase *An* was determined. The $(GlcNAc)_2$ (2.5 mg/mL) was used as substrate and hydrolyzed with pectinase *An* (1 mg/mL) in acetate buffer at 45 °C for one hour. One unit of chitobiase activity was defined as the amount of an enzyme able to hydrolyze 1 μmole of $(GlcNAc)_2$ to 2 μmole of GlcNAc per minute. The serum *Hb* and pectinase *An* was mixed by varying activity ratio between chitobiase activity of pectinase *An* and chitinase activity of serum *Hb* (**Table 2.16**). The fibrous β-chitin (300 mg) was hydrolyzed by enzyme mixing in the presence of acetate buffer pH 4.0 (1 M, 0.5mL) and NaN_3 (1%, 0.5 mL). The reaction volume was adjusted to 5.0 mL by DI-water and also incubated at 45 °C. The products of reaction were measured by HPLC.

Table 2.16 The amounts of reagents used in reactions in the production of GlcNAc in one-step by enzyme mixing

Code	Chitin (mg)	Enzyme			1% NaN_3 (mL)	Acetate buffer (mL)
		Pectinase *An* (mg, U)[b]	Serum *Hb* (mL,mU)[a]	**Mixing ratio**[c]		
POS-0.2	300	0.1, 2.14	0.6, 64.8	**0.03**	0.5	0.5
POS-1.0	300	0.5, 10.68	0.6, 64.4	**0.16**	0.5	0.5
POS-5.0	300	1.0, 21.35	0.6, 64.8	**0.33**	0.5	0.5
POS-25.0	300	5.0, 106.75	0.6, 64.8	**1.65**	0.5	0.5
POS-50.0	300	10.0, 213.50	0.6, 64.8	**3.29**	0.5	0.5

[a]Chitinase activity of serum *Hb* = 108 mU/mL (see **section 3.4**)

[b]Chitobiase activity of pectinase *An* = 21.35 mU/mg.

[c]Mixing ratio = chitobiase activity of pectinase *An*/chitinase activity of serum *Hb*.

3.9.2 Isolation of *N,N'*-diacetylchitobiose by gel-filtration chromatography

The crude product solution (1,250 mL) obtained from chitinolysis of squid pen chitin (3.6 g) with enzyme from serum *Hb* (1.58 U) from section 3.8 in **System A1** was freeze-dried to give the crude mixture (4.75 g) of GlcNAc (2.6 %, w/w) and $(GlcNAc)_2$ (12.5%, w/w). The crude product (500 mg) was dissolved in DI-water (6 mL), centrifuged and filtered through a syringe filter (0.80 μm) into a 25 mL syringe. Another portion of DI-water (6 mL) was used to rinse off all the crude product and filtered into the same syringe. The solution was mixed well before injecting into the gel-filtration column (ID 5.0 cm, Height 90.0 cm) containing Toyopearl packing materials (1.5 kg). The crude product was eluted through the column by degassed DI-water at the flow rate of 2.0 mL/min. The product was collected in test tubes with a fraction size of 2.5 mL. Each fractions were monitored for the presence of $(GlcNAc)_2$ by HPLC. The fraction containing over 98% of $(GlcNAc)_2$ were combined and freeze dried.

3.10 Production of higher molecular weight chitooligosaccharide by hydrolysis of chitosan by serum *Hb*

Chitosans (150 mg) was dissolved in acetic acid (1M, 1.0 mL), NaN_3 (1%, 1.0 mL) and water (5.0 mL). The pH of solutions were carefully adjusted with NaOH (1 M) to pH 4.0. The enzyme from the serum *Hb* (3.8 mU) was added and the reaction volume adjusted to 10.0 mL by DI-water. The mixture was incubated at 45°C. The supernatant (100 μL) was pipetted and diluted 100 fold. The mixture was added into the Ubbelohde viscometer tube. The liquid was push up above the upper level **(a)** by a balloon pump (**Figure 2.4**). The falling time of the liquid between the upper **(a)** and lower **(b)** level was measured by a stopwatch in triplicates.

After 96 hours the falling time became constant, A portion (0.3 mL) of the mixture was taken out and diluted with MeOH (0.3 mL). Acetic anhydride (0.03 mL) was added for *N*-acetylation and mixture was stirred at room temperature for one hour. The products in supernatant solution were analyzed by HPLC with similar procedure to those described previously.

The hydrolysate was dried under freeze dryer. The dried crude chitooligomer was dissolved in acetate buffer pH 3.0 (0.5 M). The molecular weights of these oligomers performed by Gel Permeation Chromatography in acetate buffer pH 3.0 (0.5 M) as an eluent. The mobile phase was flowed with flow rate 0.6 mL/min detected by refractive index detector. The injection volume was 20 μL by using Pullulan M_w 5,900-788,000 as a polysaccharide standard for calibration.

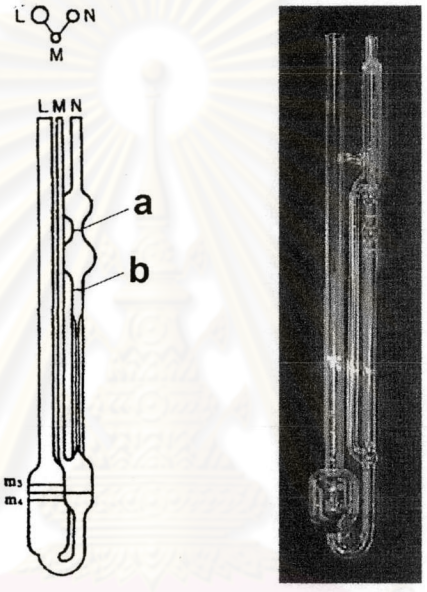

Figure 2.4 Ubbelohde viscometer