CHAPTER II

EXPERIMENTS

Instruments and Apparatus

1. High Performance Liquid Chromatography (HPLC) system: pump (Waters 600E), autosampler (Waters 917) and diode array detector (Waters 966) controlled by Millenium Program.
2. HPLC reverse phase column (Asahipak NH_2P-50, Shodex, Japan)
3. UV-Visible spectrophotometer (Jenway 6405)
4. A Millipore Alpha-Q water purification system (Millipore, USA)
5. Freeze-dryer (Freezone 77520, Benchtop, Labconco, USA)
6. Centrifugal Mill (Rector 970)
7. Centrifuge (Centaur 2, Sanyo)
8. Micropipettes P20, P200, P1000 and P5000 (Pipetman Gilson, France)
9. A pH meter (pH 3 scan, Eutech Instruments Pte Ltd, Singapore)
10. A hot-plated magnetic stirrer (Corning, USA)
11. Syringe filter (0.45 μm PTFE, Alltech)
12. Solvent membrane filters (0.45 μm cellulose, Millipore)
13. Microtubes MCT-150-C 1.5 mL clear (Axygen scientific, inc., USA)
14. Plastic Cuvettes (Polystyrene)
15. Vortex mixer (Model K-550-GE, Scientific Industries, Inc, USA)

Chemicals

1. Flake squid pen β-chitin (Ta-ming Enterprises, Thailand)
2. Powder squid pen β-chitin 3.0 μm (Koyo Chemical Co.Ltd., Japan)
3. *N*-acetyl-D-glucosamine (Fluka Chemicals, Ltd., Switzerland)
4. *N,N'*-diacetylchitobiose (Seikagaku Corporation Co.Ltd., Japan)
5. Sodium azide (Riedel-deHaën, Germany)
6. Citric acid, analytical grade (Merck, Germany)
7. Sodium hydrogen phosphate (Fluka Chemicals, Ltd., Switzerland)
8. Hydrochloric acid, analytical grade (Merck, Germany)
9. Potassium hexaferrocyanate (Merck, Germany)
10. Acetonitrile, chromatography grade (Merck, Germany)
11. Sodium chloride, analytical grade (Merck, Germany)
12. Enzyme cellulase *Acremonium celluloticus* (*Ac*) (Meiji Seika Kaisha, Ltd., Japan)
13. Enzyme chitinase *Serratia sp.* cloned (Chi 60) (Dr. Ruth Pichyang kura, Department of Biochemistry, Faculty of Science, Chulalongkorn University)
14. Sodium hydroxide, analytical grade (Merck, Germany)
15. Sodium acetate (Fluka Chemicals, Ltd., Switzerland)
16. Serva Blue G Dye (BDH chemicals Ltd, England)
17. Bovine serum albumin (BSA) (Biolab, England)
18. 95% Ethanol, analytical grade (Merck, Germany)
19. 85% Phosphoric acid, analytical grade (Carlo Erba, Italy)

General Procedure

2.1 Preparation and characterization of substrates and enzymes

2.1.1 Squid pen chitin 100 μm

The squid pen chitin (β-chitin) was purchased from Ta-ming enterprise Co., Ltd. It was ground by ultracentrifugal mill (Recter 970) at the Metallurgy and Materials Science Reseach Institute, Chulalongkorn University.

2.1.2 Colloidal chitin[62]

The concentrated hydrochloric acid was gradually added to crab chitin (50 g) in a beaker under vigorous stirring until thick viscous slurry was obtained. The sticky mixture was sonicated for 30-40 min at room temperature. One litre of ice was poured into the mixture and vigorously stirred forming instantly a fine precipitate. The slurry was kept overnight in a refrigerator at 4 °C. The precipitate was filtered off and washed with copious amount of DI-water until the filtrate became neutral.

2.1.3 Protein assaying of enzyme

2.1.3.1 Preparation of calibration curve

The standard solution of protein was prepared by pipette the bovine serum albumin (BSA) (10 mg/mL, 20 μL) into a microtube and made a total volume to 200 μL with DI-water. The standard solution at various volume was pipetted into the test tube and the McIlvaine buffer pH 6 was added into the solution to made a total volume to 100 μL **(Table 2.1)**. One mL of Bradford reagent was added and stirred by vortex. After waited for 20 min the absorption was measured at 595 nm using DI-water as a blank. The standard curve was obtained by plotting the absorbance in Y-axis against the amount of protein (μg) in X-axis.

Table 2.1 Preparation of the protein standard solutions.

Sample number	μg protein	Standard solution (1 mg/mL BSA) (μL)	0.1M McIlvaine buffer pH 6 (μL)	Bradford reagent (mL)
1	0.0	0.0	100.0	1.0
2	2.5	2.5	97.5	1.0
3	5.0	5.0	95.0	1.0
4	7.5	7.5	92.5	1.0
5	10.0	10.0	90.0	1.0
6	12.5	12.5	87.5	1.0
7	15.0	15.0	85.0	1.0
8	17.5	17.5	82.5	1.0
9	20.0	20.0	80.0	1.0

2.1.3.2 Measurement of the protein amount in the Chi 60

The amount of protein was determined by following Bradford method.[63] The protein solution was added with the McIlvaine buffer pH 6 to made a total volume to 100 μL. Bradford reagent (1 mL) was added and the mixture was stirred by vortex and waited for 20 min. The amount of protein was measured by UV-visible spectrophotometer at 595 nm. The amount of protein was calculated by comparison with the standard curve of BSA protein prepared in the previous section **(Figure A3)**.

2.1.4 Chitinase activity assaying

2.1.4.1 Preparation of the calibration curve

The stock solutions **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** (5.02 mM) and stock solution **B** (2.51 mM) were diluted to various desired concentration of the standard solutions **(Table 2.2)**.

Table 2.2 Concentration of GlcNAc and standard solutions 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

The colloidal chitin (11.4 mg/mL,50 μL) was added in each standard solution and made the volume to 1.5 mL with DI-water. The control tube was added only colloidal chitin 50 μL into DI-water 1.5 mL. The coloring reagent solution ($K_3Fe(CN)_6$ (0.05% (w/v), 2mL) in 1 M Na_2CO_3) were mixed into each standard solution including the control tube and heated in boiling water for 15 min in the test tube covered with aluminum foil. After cooling, the mixtures were centrifuged at 2500 rpm for 15 min to remove the residues. 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 sample – Absorbance of the standard sample) in Y-axis against the amount of GlcNAc (μmole) in X-axis.

2.1.4.2 Activity assaying

Chitinase activities were assayed by measuring the amount of reducing sugar produced from the hydrolysis of colloidal chitin using a modified Schales method[64]

The mixture composed of colloidal chitin (0.05 mL, 50 mg/mL), DI-water (1.15 mL) and the desired amount of chitinase in acetate-phosphate buffer pH 3 (0.5 M, 0.3 mL) were transferred into a dry clean test tube. This mixture was incubated at 37 °C for 15 min. After the incubation period, The coloring reagent (2mL) was mixed with the mixture and heated in boiling water for 15 min in the test tube covered with

aluminum foil. After cooling to room temperature, the mixture was centrifuged at 2500 rpm for 15 min to remove the residues.

For the control tube, the solution of enzyme chitinase was boiled in boiling water for 15 min before it was pipetted into a dry test tube and followed the same procedure as described above. The absorbance at 420 nm was measured with a UV-visible spectrophotometer by using DI-water as a reference. Both mixtures and controls were performed in two replicates. The activity unit (U) per volume (mL) of the enzyme was calculated from the difference of the absorbance (ΔA) between the mixture (A_1) and the control (A_0) according to the following equation:

$$\text{Activity (U)} = \mu\text{mole of reducing sugar/ t /mL of enzyme}$$

$$= (\Delta A/1.0724)/15/0.3$$

The factor of 1.0724 was the slope of the calibration line **(section 2.1.4.1, Figure A4)** and t was the incubation time of this experiment.

One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 μmole of GlcNAc equivalent per min. Specific activity was defined as units per milligram of enzyme sample.

For the assaying of chitinase from Chi 60, the mixture composed of colloidal chitin (0.05 mL, 50 mg/mL), DI-water (0.95 mL) and the desired amount of chitinase in a 0.5 M acetate-phosphate buffer pH 6 (0.3 mL) was put in a test tube and followed the same procedure as described above.

2.2 Study of hydrolytic products by HPLC

2.2.1 Preparation of calibration curve for *N*-acetyl-D-glucosamine (GlcNAc)

The exact weight of dried GlcNAc ($C_8H_{15}NO_6$ = 221.21) were dissolved in Milli-Q water (5mL) 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	4.8400	**GlcNAc** (24.2 mg) + water (5 mL)
B	2.4800	**GlcNAc** (12.4 mg) + water (5 mL)
C	1.2100	**A** (0.5 mL) + water (1.5 mL)
D	0.4840	**A** (0.2 mL) + water (1.8 mL)
E	0.2420	**A** (0.2 mL) + water (3.8 mL)
F	0.1210	**A** (0.1 mL) + water (3.9 mL)
G	0.1860	**B** (0.225 mL) + water (2.775 mL)
H	0.0620	**B** (0.1 mL) + water (3.9 mL)
I	0.0186	**G** (0.2 mL) + water (1.8 mL)
J	0.0093	**I** (0.2 mL) + water (1.8 mL)

Each standard (0.300 mL) was mixed with acetonitrile (0.700 mL) and filtered through a 0.45 μm PTFE membrane filter. The standard solutions (20 μL) were analyzed by HPLC (column: Shodex Asahipak NH_2P-50; mobile phase: 30:70 water:acetonitrile; flow rate: 1.00 mL/min; detection: UV at 210 nm). 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.

2.2.2 Preparation of calibration curve for *N,N'*-diacetylchitobiose ($(GlcNAc)_2$)

The calibration curve for $(GlcNAc)_2$ ($C_{16}H_{28}N_2O_{11}$ = 424.42) was obtained by using the similar procedure described for GlcNAc except for that $(GlcNAc)_2$ was used as a standard in place of GlcNAc **(Table 2.4)**.

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

Standard solution	Concentration (mg/mL)	Preparation method
A	1.600	**$(GlcNAc)_2$** (2.4 mg) + water (1.5 mL)
B	1.000	**$(GlcNAc)_2$** (2.1 mg) + 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)

2.2.3 Analysis of the hydrolytic products

After the designated time, the hydrolysates of each reaction mixture (100 μL) was pipetted into a 2 mL plastic capped microtube 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 min. The supernatant (300 μL) was pipetted and mixed with acetonitrile (700 μL). The solution was filtered through a 0.45 μm PTFE membrane filter before injected into the HPLC. The GlcNAc and $(GlcNAc)_2$ were detected at retention times of 5.6 and 6.5 min, respectively. The peak areas were used to calculate the amount of the GlcNAc and $(GlcNAc)_2$ according to the following equations:

$$[GlcNAc]\ (mM) = \frac{\text{peak area}}{353.29} \times \text{dilution factor}$$

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

The factors of 353.29 and 495.54 were obtained from the slope of the calibration lines of GlcNAc and $(GlcNAc)_2$, respectively **(Figures A1-A2)**.

2.3 Study for the optimum condition for the enzymatic hydrolysis with cellulase *Acremonium cellulolyticus*

2.3.1 Concentration of chitin

The concentration of chitin was varied while the concentration of chitinase from cellulase *Acremonium cellulolyticus* (*Ac*) was fixed at 260 mU/mL. The chitin substrate and the enzyme were weighed into a plastic-capped vial. Citrate-phosphate (McIlvaine) buffer solution pH 3 (0.5 M, 1.0 mL) and sodium azide solution (1%, 0.5 mL) were added. The reaction volume was adjusted to 5.0 mL with Milli-Q water **(Table 2.5)**. The reaction mixture was incubated at 37 °C with continuous stirring. The reactions were monitored for four consecutive days.

Table 2.5 The amounts of reagents for hydrolysis of chitin with enzyme chitinase from cellulase *Ac*.

Chitin type	**wt. of chitin (mg)**	wt. of cellulase *Ac* (mg)	Milli-Q water (mL)
Powder 3.0 μm	**50**	10	3.5
	100	10	3.5
	150	10	3.5
	200	10	3.5
	250	10	3.5
	300	10	3.5
	400	10	3.5
	500	10	3.5
	1000	10	3.5
Fibrous chitin 50 x 25 μm	**50**	10	3.5
	100	10	3.5
	150	10	3.5
	300	10	3.5

2.3.2 Concentration of chitinase

In this experiment, the concentration of chitinase cellulase *Ac* was varied while the concentration of chitin was fixed at 80 mg/mL for powder chitin 3.0 μm and 60 mg/mL for fibrous chitin. The chitin substrate and the enzyme were weighed into a plastic-capped vial. The McIlvaine buffer solution (0.5 M, 0.6 mL) and sodium azide solution (1%, 0.3 mL) were added. The reaction volume was adjusted to 3.0 mL by Milli-Q water **(Table 2.6)**. The reaction mixture was incubated at 37 °C with continuous stirring. The reactions were monitored on the 1st, 3rd, 5th and 9th dates by HPLC.

Table 2.6 The amounts of reagents used in the study for the optimum concentration of chitinase from cellulase *Ac*.

Chitin type	wt. of chitin (mg)	**wt. of cellulase *Ac* (mg)**	Milli-Q water (mL)
Powder 3.0 μm	240	**15**	2.1
	240	**60**	2.1
	240	**90**	2.1
	240	**120**	2.1
Fibrous chitin 50 x 25 μm	180	**15**	2.1
	180	**30**	2.1
	180	**60**	2.1
	180	**120**	2.1

2.3.3 pH of the reaction solution

pH dependence of chitinolytic activity of cellulase *Ac* on β-chitin was studied by varying the pH from 2.0 to 5.0 using citrate and citrate-phosphate buffers. The concentration of both chitin and enzyme in reaction mixture were 10 mg/mL. The citrate buffer was used for pH 2.0 and citrate-phosphate buffer was used for pH 3.0-5.0. After incubation at 37 °C for 1, 3 and 6 days, the reaction mixtures were sampled and analyzed by HPLC.

2.3.4 Concentration of buffer

The hydrolysis of fibrous β-chitin with cellulase *Ac* were carried out in various concentration of McIlvaine buffer pH 3. The chitin (300 mg) was placed in a screw-capped vial. The mixture of cellulase *Ac* (150 mg), NaN_3 (1%, 0.5 mL) and buffer pH 3 with appropriate concentration was added and made a volume to 5.0 mL by milli-Q water (**Table 2.7**). The reaction mixture was incubated at 37 °C with continuous stirring. The reactions were monitored on the 1st, 3rd, 5th and 9th dates by HPLC.

Table 2.7 The amounts of reagents used in the reaction in the study for suitable concentration buffers.

Chitin	Wt. of cellulase *Ac*	McIlvaine buffer (0.5 M)		Milli-Q water
(mg)	(mg)	Vol. (mL)	**Conc. (M)**	(mL)
300	150	0.0	**0.0**	4.5
300	150	0.5	**0.05**	4.0
300	150	1.0	**0.1**	3.5
300	150	2.0	**0.2**	2.5
300	150	4.0	**0.4**	0.5

2.3.5 Temperature

The squid chitin powder (3.0 μm, 240 mg) and cellulase *Ac* (90 mg) were weighed into four vials. The McIlvaine buffer solution pH 3 (0.5 M, 0.6 mL), NaN_3 solution (0.3 mL) and Milli-Q water (2.1 mL) were added. The reaction mixture were incubated at various temperature (30, 37, 45 and 55 °C) with continuous stirring. After incubation for 1, 3, 5 and 9 days, each reaction mixture was sampled and then analyzed by HPLC.

2.3.6 Enzyme affinity technique

The suspension of squid chitin powder (50mg/mL, 3 mL), cellulase *Ac* (50 mg/mL,1 mL) and the McIlvaine buffer solution pH 3 (0.5 M, 1mL) were placed into four clean vials and were shaken to allow good mixing. The mixture was chilled in a refrigerator for 1 hr, 4 hr, and 24 hr. After chilling, the mixture was centrifuged for 10 min. The supernatant (2.5 mL) was withdrawn and freshly chilled buffer (2.5 mL) was

added. This process was repeated 3 times. Then, chilled buffer (2.25 mL) and NaN_3 solution (1%, 0.25 mL) were added. The reaction mixture was incubated at 37 °C with continuous stirring. The reactions were monitored on the 1st, 2nd, 3rd and 4th dates by HPLC.

2.4 Study for the optimum condition for the enzymatic hydrolysis with chitinase *Serratia sp.* cloned (Chi 60)

2.4.1 Concentration of chitin

The concentration of chitin was varied while the concentration of chitinase Chi 60 was fixed at 19.08 mU/mL. The chitin substrate and the enzyme were weighed into a plastic-capped vial. Citrate-phosphate (McIlvaine) buffer solution pH 6 (0.5 M, 1.0 mL) and sodium azide solution (1%, 0.5 mL) were added. The reaction volume was adjusted to 5.0 mL with Milli-Q water **(Table 2.8)**. The reaction mixture was incubated at 37 °C with continuous stirring. The reactions were monitored on the 1st, 2nd, 3rd, 4th and 7th dates by HPLC.

Table 2.8 The amounts of reagents for hydrolysis of chitin with enzyme chitinase from chi 60

Chitin type	**wt. of chitin (mg)**	wt. of Chi 60 (mg)	Milli-Q water (mL)
Fibrous chitin	**50**	10	3.5
100 x 50 μm	**100**	10	3.5
	150	10	3.5
	300	10	3.5

2.4.2 Concentration of chitinase

In this experiment, the concentration of Chi 60 was varied while the concentration of chitin was fixed at 30 mg/mL for squid pen chitin 50 × 25 μm. The chitin substrate and the enzyme were weighed into a plastic-capped vial. The McIlvaine buffer solution pH 6 (0.5 M, 0.6 mL) and sodium azide solution (1%, 0.3 mL) were added. The reaction volume was adjusted to 3.0 mL by Milli-Q water

(Table 2.9). The reaction mixture was incubated at 37 °C with continuous stirring. The reactions were monitored on the 1st, 3rd, 5th and 9th dates by HPLC.

Table 2.9 The amounts of enzyme and water needed in each reaction.

Chitin type	wt.of chitin (mg)	**chi 60 (mU)**	Milli-Q water (mL)
Fibrous chitin	90	**4.00**	1.539
50 × 25 μm	90	**5.00**	1.399
	90	**6.55**	1.186
	90	**9.32**	0.800
	90	**11.00**	0.565
	90	**13.00**	0.286
	90	**15.00**	0.007
	90	**30.00**	1.769
	90	**50.00**	1.548
	90	**100.00**	0.997
	90	**150.00**	0.445
	90	**250.00**	1.171
	90	**350.00**	0.799

2.4.3 pH of the reaction solution

For Chi 60, the pH was varied from 3.0-7.0 by using citrate-phosphate buffer solution. The fibrous chitin (100×50 μm, 50 mg) was weighed into a plastic-capped vial. The mixture of Chi 60 (4.77 mU), NaN_3 (1%, 0.5mL) and buffer (0.5 M, 1 mL) were added and the volume was made to 5.0 mL by Milli-Q water. The reaction mixture was incubated at 37 °C with continuous stirring. The reactions were monitored for four consecutive days by HPLC.

2.4.4 Concentration of buffer

The hydrolysis of fibrous β-chitin with Chi 60 was carried out in various concentration of McIlvaine buffer pH 6. The chitin (90 mg) was placed in a screw-capped vials. The mixture of Chi 60 (50 mU), NaN_3 (1%, 0.3 mL) and buffer pH 6

with designated concentration was added and the volume was made to 3.0 mL by milli-Q water (**Table 2.10**). The reaction mixtures were incubated at 37 °C with continuous stirring. The reactions were monitored on the 3rd , 5th and 9th dates of the incubation by HPLC.

Table 2.10 The amounts of reagents used in the reaction in the study for optimum buffer concentration.

Chitin	Wt. of cellulase *Ac*	McIlvaine buffer (0.5 M)		Milli-Q water
(mg)	(mg)	Vol. (mL)	**Conc. (M)**	(mL)
90	50	0.0	**0.0**	2.45
90	50	0.3	**0.05**	2.15
90	50	0.6	**0.1**	1.85
90	50	1.2	**0.2**	1.25
90	50	2.4	**0.4**	0.05

2.4.5 Temperature

The chitin 50×25μm (90 mg) was weighed into a plastic-capped vial. The mixture of Chi 60 (150 mU), NaN_3 (1%, 0.3mL) and McIlvaine buffer pH 6 (0.5 M, 0.6 mL) were added and the volume was made to 3.0 mL by Milli-Q water. The reaction mixture was incubated at various temperature (30, 37, 45, 50 and 55 °C) with continuous stirring. The reactions were monitored on the 1st, 2nd, 3rd, 6th and 8th dates by HPLC.

2.4.6 Enzyme affinity technique

For Chi 60, the chitin (90 mg) was weighed into four test tubes. The mixture of Chi 60 (150 mU) and cold McIlvaine buffer pH 6 (0.5 M, 0.6 mL) were added into the tube and were shaken to allow good mixing. The mixture was chilled in a refrigerator for 1 hr, 3hr, and 24 hr. After chilling, the mixture was centrifuged for 10 min. The supernatant (1.5 mL) was withdrawn and chilled buffer (1.5 mL) was added. The process was repeated 3 times. Then, cool buffer (1.2 mL) and 1% NaN_3 solution (0.3 mL) were added. The reaction mixture was incubated at 37 °C with continuous stirring. The reactions were monitored on the 1st, 2nd, 4th, 6th and 8th dates by HPLC.

2.4.7 Preparative scale preparation of *N,N'*-diacetylchitobiose

The fibrous chitin (50×25μm, 2 g) was weighed into a 125 mL erlenmeyer flask. The mixture of Chi 60 (30 mU), NaN_3 (1%, 6.7 mL) and McIlvaine buffer pH 6 (0.5 M, 13.4 mL) were added and the volume was made to 67.0 mL by Milli-Q water. The reaction mixture was incubated at 37 °C with continuous stirring. The reactions were monitored after 1, 2, 4 and 6 days of incubation by HPLC.

2.4.8 Hydrolysis of $(GlcNAc)_2$

Dried $(GlcNAc)_2$ (1.5 mg, 3.59 μmole) was weighed into a microtube. The enzyme (10 mU), NaN_3 (1%, 0.1 mL) and McIlvaine buffer (0.5 M) were added and the total volume of the reaction were adjusted by Milli-Q water to 1.0 mL **(Table 2.11).** The reactions were stirred continually by a magnetic stirrer and incubated at 37 °C. The reactions were monitored after 1 hr, 3hrs, 5hrs and 24 hrs of incubation by HPLC.

Table 2.11 The amounts of reagents used in the reaction in the study of hydrolysis of $(GlcNAc)_2$

	$(GlcNAc)_2$	Enzyme	McIlvaine buffer		1% NaN_3	Milli-Q
	(mg, μmole)	(mU)	pH	Vol. (mL)	(mL)	water (mL)
Cellulase *Ac*	1.5, 3.59	10.0	3.0	0.2	0.1	0.7
Chi 60	1.5, 3.59	10.0	6.0	0.2	0.1	0.23