CHAPTER III

RESULTS AND DISCUSSIONS

1. Enzyme from serum *Hb*

Latex of the rubber tree is the cytoplasm of specialized cells known as laticifers. The major components of latex are rubber particles, cytosol and organelles called lutoids. The latter are of vacuolar origin and contain several hydrolases. Upon centrifugation at 54,000 g for 40 min, the fresh latex is divided into three layers: a layer of rubber particles, a layer of cytosol (C-serum), and lutoid body fraction (B-serum). However, in industrial process, the latex rubber used in this work was centrifuged at 10,000 g and separated into two layers, a layer of rubber particle (concentrated rubber) and a layer of B-serum and C-serum (skim rubber). The skim rubber, which consisted of ~5% rubber particles, was added with H_2SO_4 (50%) to pH 5 and the serum *Hb* was separated and drained to the waste well. This yellow solution serum *Hb* was filtered and stored at 4 °C for further use in all experiments throughout this thesis.

2. β-Chitin as substrate

In this study, the squid pen chitin from local chitin-chitosan producer was made available to be used as a main raw material for studying hydrolysis reaction. The flake squid pen chitin was prepared as substrate by grinding with a centrifugal mill to give fluffy fibrous chitin with an average diameter about 50 μm and length about 100 μm (**Figure 3.1**).

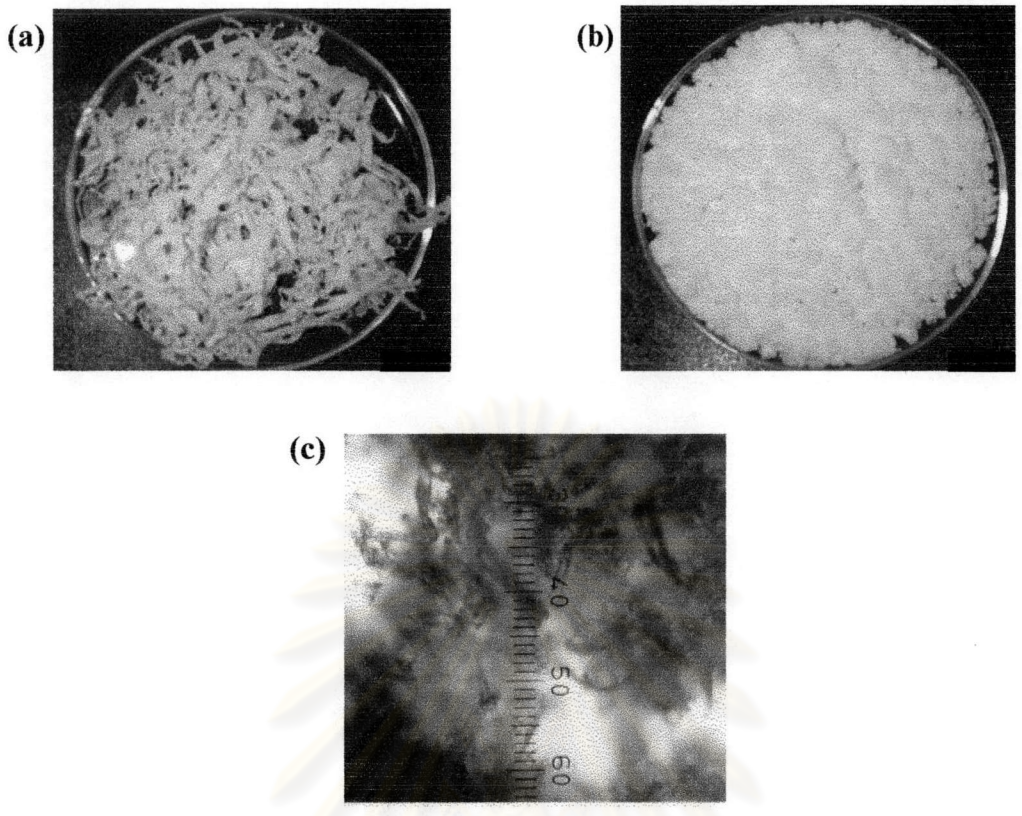


Figure 3.1 The squid pen chitin, (a) flake chitin, (b) fibrous-β-chitin 100×50 μm (1×), (c) fibrous β-chitin under optical microscope (15.5×) (1 gap = 0.03 mm).

Degree of acetylation (%DA) of all chitin substrates (fibrous β-chitin, β-chitin powder, and colloidal chitin) were determined from the elemental analysis (EA) data (**Figure A6**). The results showed that both fibrous β-chitin and β-chitin powder have %DA over 90%. In contrast, the colloidal chitin has %DA only around 71% (**Table 3.1**). The low %DA of the colloidal chitin may be attributed to partial deacetylation during the preparation of this substrate under acidic condition.

Table 3.1 The results of C, H, O, N elemental analysis

% element	% C	% H	% N	C/N ratio	**%DA**[a]
Fibrous β-chitin	42.309	6.661	6.304	6.711	**91**
β-chitin powder	42.468	6.730	6.234	6.812	**97**
colloidal chitin	43.896	6.453	6.892	6.369	**71**

[a]Calculated from the linear relationship between %DA and C/N ratio (see appendixes A, **Figure A6**).

3. Characterization of the enzyme in serum *Hb*

3.1 The determination of protein content in serum *Hb*

The serum *Hb* (lot. Nov 9 2001) from Pan Asia Biotechnology Co.,Ltd., (Rayong, Thailand) was analyzed for its protein content by settling protein with ISO/FDIS procedure[89] following Lowry's method.[90] The analysis showed that the serum *Hb* contained 6.0 mg protein per 1 mL of serum *Hb* solution.

3.2 The chitinolytic activity of enzyme in serum *Hb*

The chitinolytic activity was assayed by measuring the amount of reducing sugars produced in the enzymatic hydrolysis, based on Schales method, using colloidal chitin as the substrate in sodium acetate buffer (pH 4.0, 0.1M) at 37°C for 30 min of incubation period. The activity of enzyme was 108 mU in serum *Hb* 1 mL where one unit (U) of enzyme activity was defined as the amount of an enzyme able to produce the product with reducing ability equal to 1 μmole of GlcNAc per min. The specific enzyme activity defined as unit per mg of protein, of the enzyme serum *Hb* was 18 mU/mg protein. The enzyme in serum *Hb* displayed chitinolytic activity comparable to some other crude enzymes from fungi reported previously.[92]

4. Study for optimum condition in the hydrolysis of chitin and serum *Hb*

4.1 The effect of enzyme:chitin ratio

The effect of the ratio between the enzyme and chitin on the production of $(GlcNAc)_2$ and GlcNAc was investigated in this experiment. The ratio of the enzyme/substrate was varied as 0.11, 0.52, 1.08, 1.61, and 2.20 mU/mg by fixing the amount of chitin substrate at 20 mg/mL. The results showed that the yields of both GlcNAc and $(GlcNAc)_2$ increased with the augmentation of enzyme/chitin ratio (**Figure 3.2 and Table B1**), especially when the ratio was increased from 0.11 mU/mg to 1.08 mU/mg. However, when the enzyme/chitin ratio was increased over 1.08 mU/mL, only sluggish rise of the products was observed. Therefore, the optimum ratio of enzyme/chitin for effective use of the serum *Hb* should be in the range of 0.11-1.08 mU/mg. On that account, the enzyme/chitin ratio of 0.22 mU/mg was chosen for further study as this ratio also provided appropriate concentration of the products to be effectively analyzed by HPLC over the course of the hydrolysis.

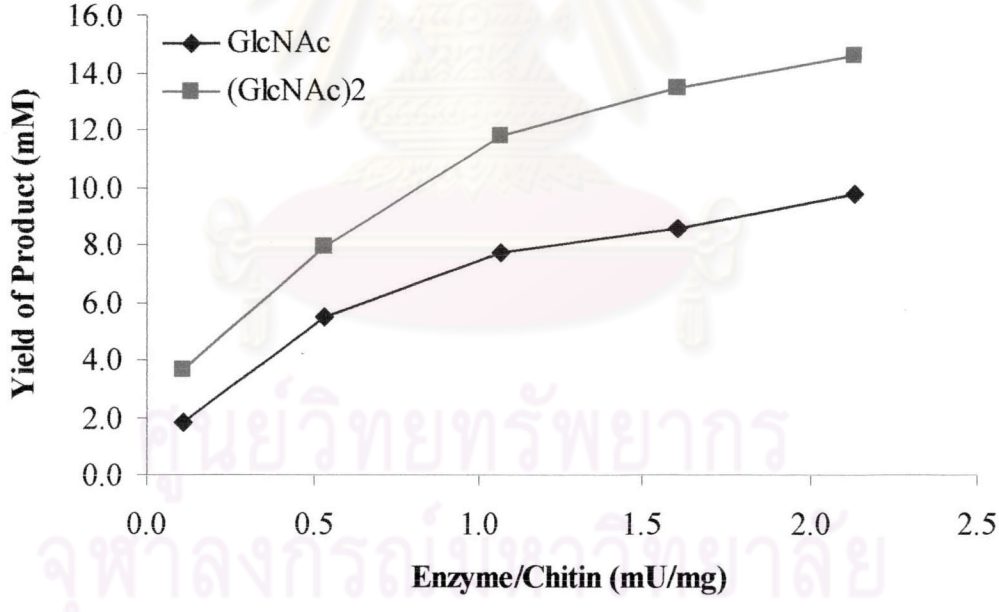


Figure 3.2 The effect of enzyme/chitin ratio on the chitinolysis. Condition; [β-chitin] = 20 mg/mL, NaOAc pH 4.0 (0.05M), 37 °C, 8th days

4.2 The effect of concentration of chitin

The most obvious means to increase the product yield and concentration is to raise the concentration of chitin substrate while maintaining the enzyme/chitin ratio at 0.22 mU/mg. As expected, the yield of both saccharides went up with the increasing concentration of chitin up to 60 mg/mL (**Figure 3.3 and Table B2**) where the stirring became ineffective due to the high content of the solid chitin. Therefore, the concentration of chitin at 60 mg/mL was chosen for further study.

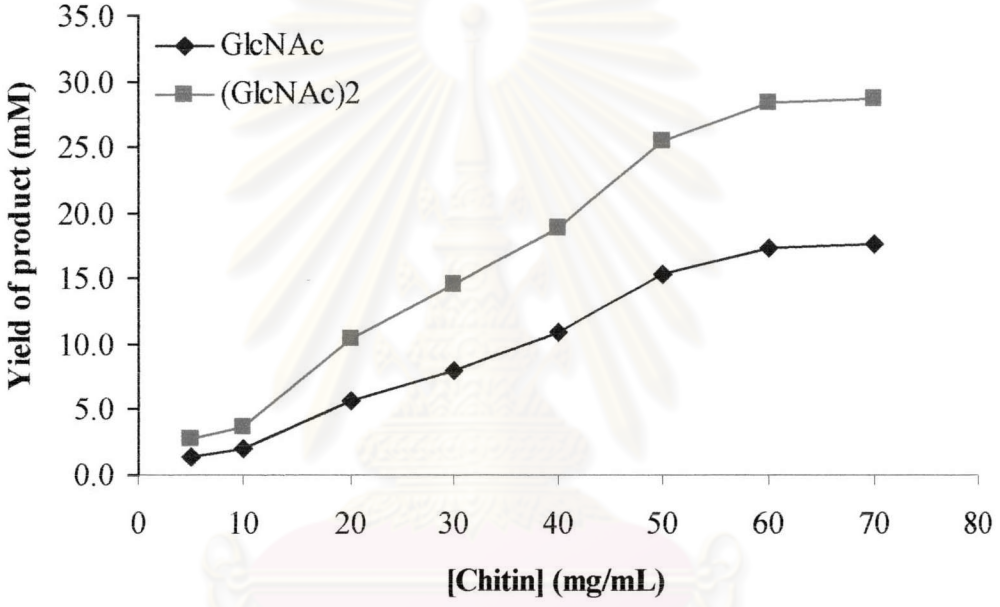


Figure 3.3 The effect of concentration of chitin on the chitinolysis. Condition; enzyme/chitin ratio = 0.22 mU/mg, NaOAc pH 4.0 (0.05M), 37 °C, 8th days.

4.3 The effect of pH of the reaction solution

The pH dependence of chitinolytic enzyme was investigated. The hydrolysis reactions were incubated at 37 °C in buffers with pH ranging from 2.0-5.5 at chitin concentration of 60 mg/mL and enzyme/chitin ratio of 0.22 mU/mL. The amino sugars produced from chitinolysis at different pH were analyzed by HPLC. It was apparent that chitinolytic enzyme in the serum *Hb* was acidophilic with broad optimum pH range between 2.0-4.0 (**Figure 3.4 and Table B3**). This result indicated that the pH dependence of the enzymes in serum *Hb* was rather different from that of hevamine, the reported chitinase found in serum *Hb*. Hevamine was reported to show the optimum pH in the range of 4.0 to 6.0.[81] This crude serum *Hb* may consist of other chitinolytic enzymes which are active in the more acidic range.

As serum *Hb* obtained from industry has its original pH about 3.5-4.0, it was convenient to adjust the pH of this serum with acetate buffer pH 4.0. The subsequent experiments were thus carried out in acetate buffer pH 4.0 (0.1M).

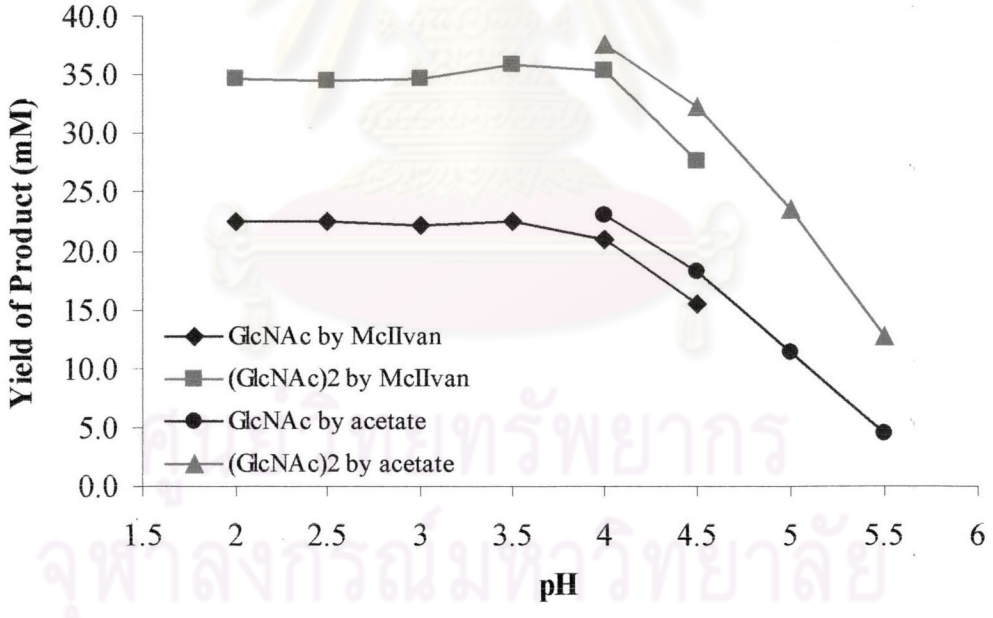


Figure 3.4 The effect of pH on the chitinolysis. Condition; [β-chitin] = 60 mg/mL, enzyme/chitin ratio = 0.22 mU/mg, 37 °C. The pH of solution were 2.0 - 4.5 (citrate phosphate), and 4.0-5.5 (sodium acetate), 8th days

4.4 The effect of types of buffer

The effect of types buffer on chitinolytic activity was also examined. Five types of buffer with pH 4.0 (sodium acetate, potassium acetate, citrate phosphase (McIlvan), sodium citrate, and potassium hydrogen phthalates (KHP)) at the same concentration (0.1M) were used. The results illustrated that no significant different effect of the types of buffers on the chitinolytic activity of the enzyme complexes in the serum *Hb* (**Figure 3.5 and Table B4**). Sodium acetate was thus chosen for further condition optimization due to its low cost.

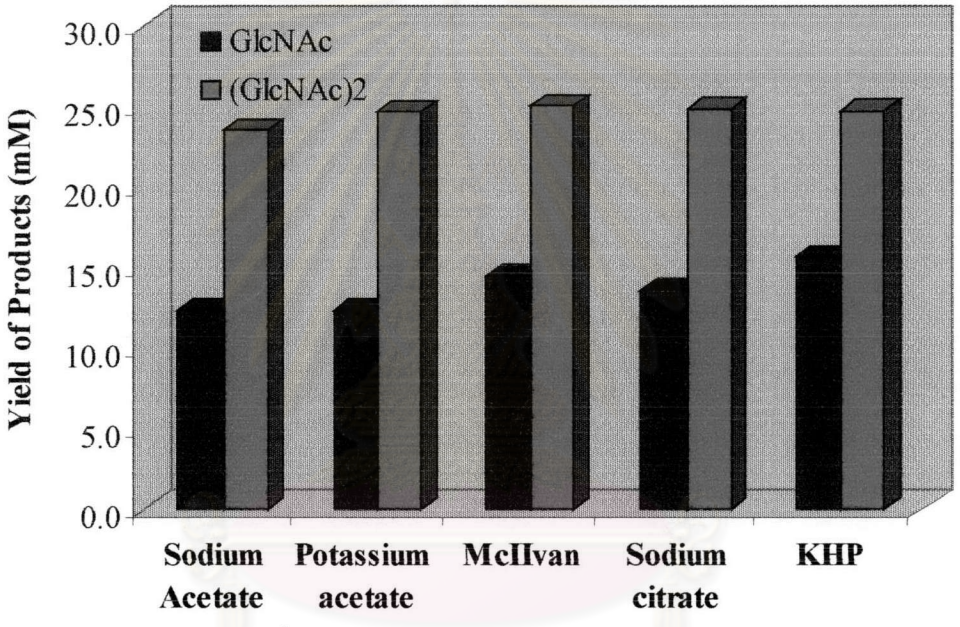


Figure 3.5 The effect of type of buffer on the chitinolysis. Condition; [chitin] = 60 mg/mL, enzyme/chitin ratio = 0.22 mU/mg, buffer pH 4.0 (0.1 M), 37 °C, 4th days.

4.5 The effect of temperature

In order to obtain the optimum temperature for the hydrolysis of chitin with the enzyme from the serum *Hb*, the chitinolysis was carried out at 30, 37, 45, and 55 °C in acetate buffer pH 4 (0.1M) with chitin concentration of 60 mg/mL and enzyme/chitin ratio of 0.22 mU/mg. The chitinolytic activity was analyzed by HPLC in the form of GlcNAc and $(GlcNAc)_2$ produced from the reaction. The chitinolytic activity dramatically increased with increasing temperature and reached the maximum yields of the products at 45 °C (**Figure 3.6 and Table B5**). Beyond this optimum temperature the activity dropped by approximately 15%.

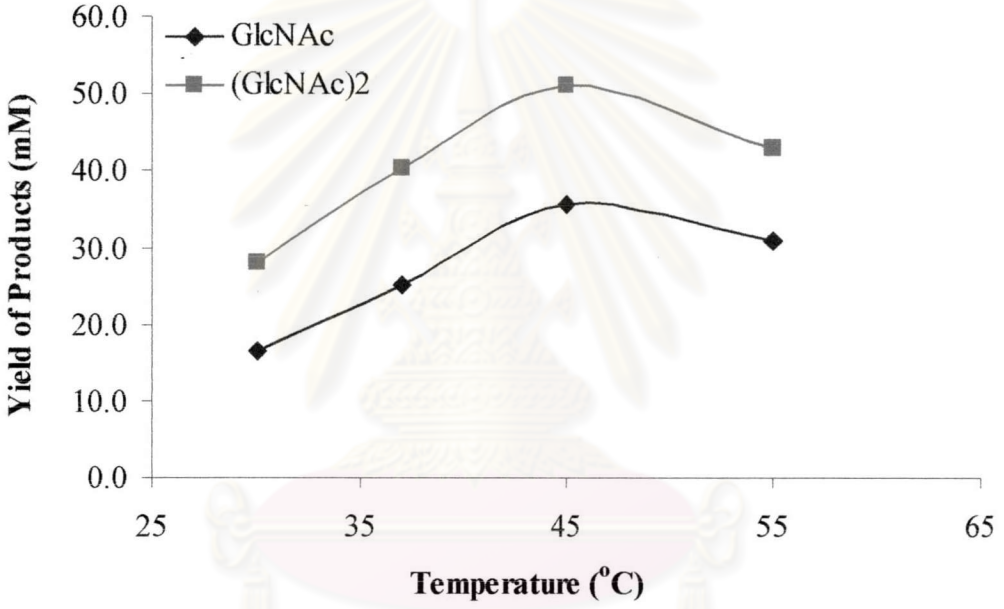


Figure 3.6 The effect of temperature on the chitinolysis. Condition; [chitin] = 60 mg/mL, enzyme/chitin ratio = 0.22 mU/mg, NaOAc pH 4.0 (0.1 M), 8th days.

4.6 The effect of concentration of buffer

The concentration of buffer was varied from 0.0 M to 0.4 M at the optimum pH. The concentration of products increased when the concentration of buffer was increased from 0.0 M to 0.1 M and became relatively constant at the concentration of buffer above 0.1 M (**Figure 3.7 and Table B6**). The results suggested that 0.1 M was the minimum buffer concentration required for maintaining the pH throughout the course of the hydrolysis. Subroto *et al.* reported that the chitinolytic activity of hevamine was depended on the concentration of salt. They found that the activity increased when the concentration of salt was increased but the activity was dropped when the concentration reached 0.4 M.[81]

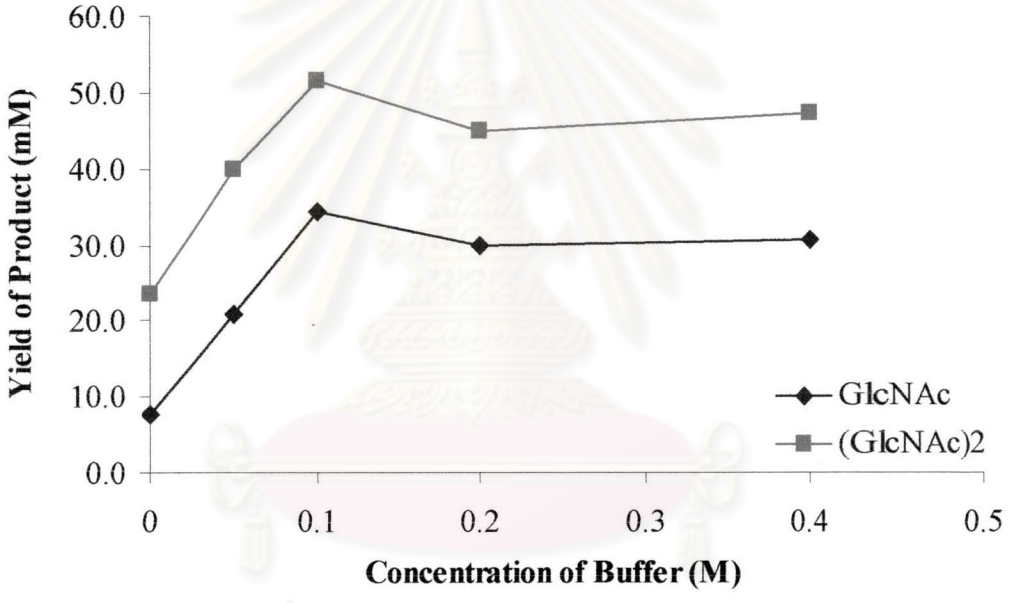


Figure 3.7 The effect of concentration of buffer on the chitinolysis. The buffer concentrations were 0.0 M–0.4 M, [chitin] = 60 mg/mL, enzyme/chitin ratio = 0.22 mU/mg, NaOAc pH 4.0, 45 °C, 8th days.

4.7 Substrate dependence

Chitin with four different substrate preparations; two types of fibers, one type of powder, and colloidal chitin was investigated. The two types of fibers used were different in their dimension; one was 50 μm in diameter and 100 μm in length and the other was 25 μm in diameter and 100 μm in length. The chitin powder was 3.0 μm in diameter. The hydrolysis of these substrates at the optimum condition revealed that the yields of both GlcNAc and $(GlcNAc)_2$ in the first day seem to increase with decreasing sizes of the substrate (**Figure 3.8 and Table B7**). However, as the reaction proceeded to the 8th day, the difference in the production yields became minimal. Interestingly though, the colloidal chitin showed disappointingly lower of both GlcNAc and $(GlcNAc)_2$ than that of the fibrous and particle chitin (**Figure 3.9 and Table B7**). This result could be attributed to the lower degree of acetylation of colloidal chitin. (see also **Table 3.1**)

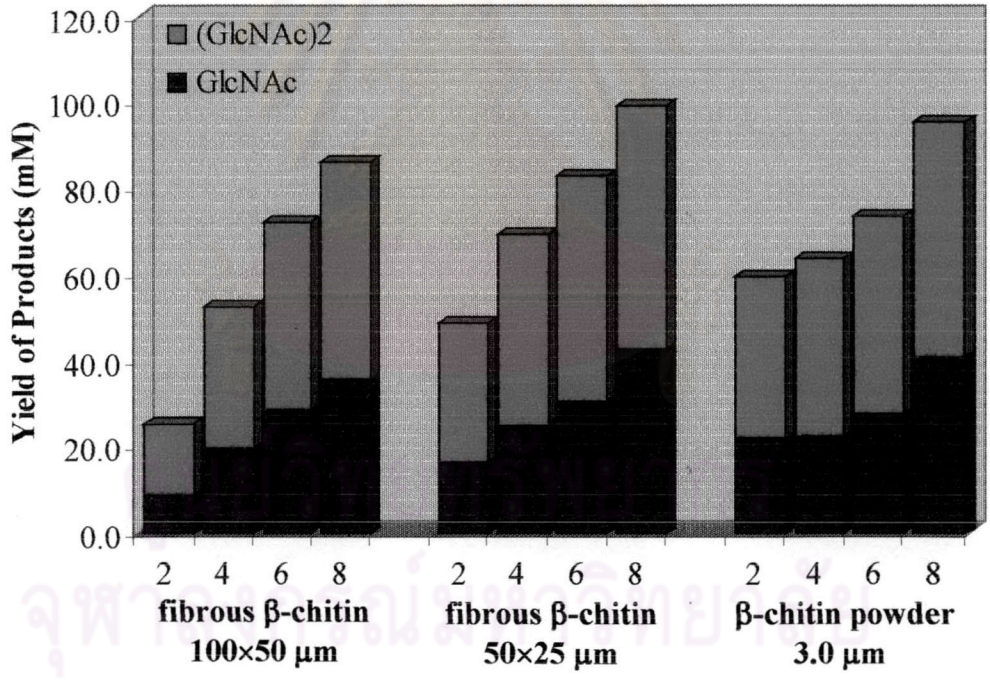


Figure 3.8 The yield of GlcNAc and $(GlcNAc)_2$ in the study of substrate dependence in the chitinolysis reaction. Condition; [chitin] = 60 mg/mL, enzyme/chitin ratio = 0.22 mU/mg, NaOAc pH 4.0 (0.1M), 45 °C, 2-8 days.

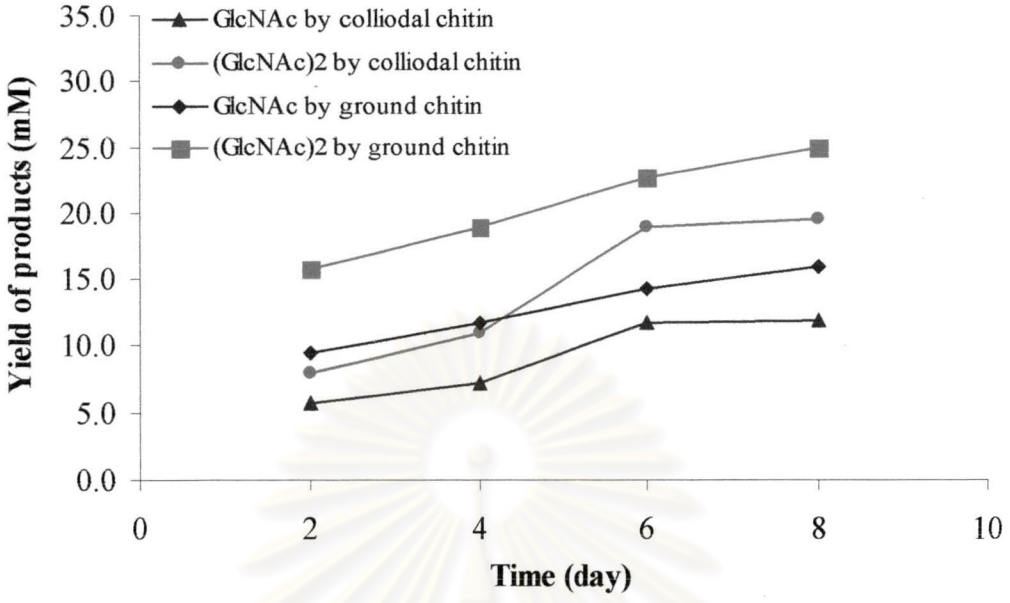


Figure 3.9 The yield of GlcNAc and $(GlcNAc)_2$ in the hydrolysis of fibrous β-chitin and colloidal chitin. Condition; [chitin] = 20 mg/mL, enzyme/chitin ratio 0.22 mU/mg, NaOAc pH 4.0 (0.1M), 45 °C, 2-8 day.

4.8 The hydrolysis time course

At the 8th day, HPLC yields of GlcNAc and $(GlcNAc)_2$ from the hydrolysis reaction of β-chitin (300 mg) with crude enzyme serum *Hb* at the optimum condition were 38 mg and 109 mg, respectively, that represented 11.63% and 35.8% the starting chitin. The total conversion of chitin to GlcNAc and $(GlcNAc)_2$ was thus around 48%. The reaction was virtually terminated after 8 days (**Figure 3.10**).

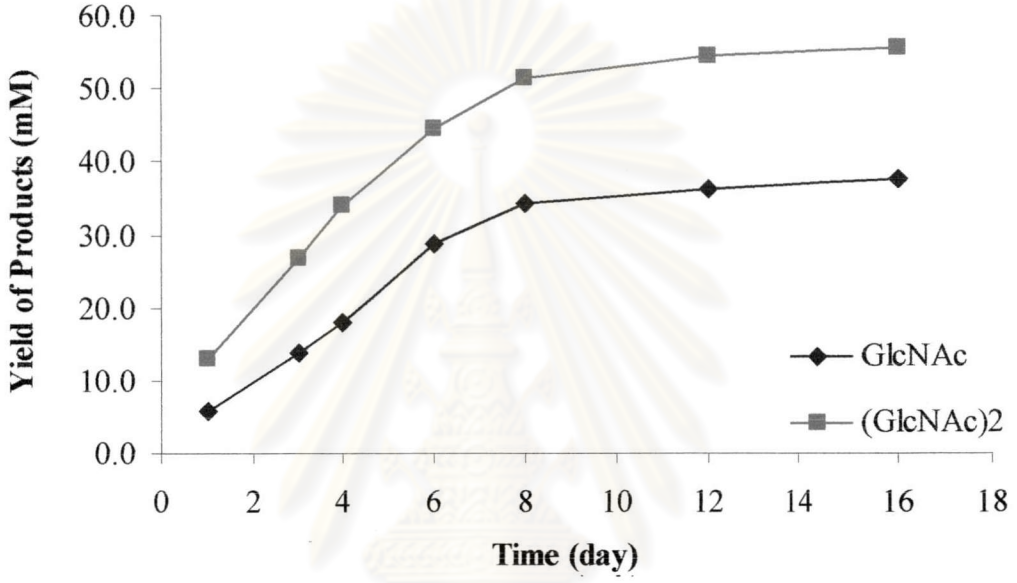


Figure 3.10 The time course of GlcNAc and $(GlcNAc)_2$ produced from the enzyme hydrolysis of fibrous β-chitin. Condition; [chitin] = 60 mg/mL, enzyme/chitin ratio = 0.22 mU/mg, NaOAc pH 4.0 (0.1 M), 45 °C, 1-16 days.

In most of the enzymatic hydrolysis reactions, there are two major causes for the reaction to stop prematurely: an enzyme denature and a product inhibition. Therefore, the enzyme/chitin ratio used in the hydrolysis was increased from 0.22 mU/mg to 0.88 mU/mg, initial production of both GlcNAc and $(GlcNAc)_2$ during the first four days tended to increase with the ratio but the final production yields on the eighth day were virtually the same (**Figure 3.11 and Table B8**). These results suggested that enzyme denature was not likely to be the cause for limiting the final yields.

GlcNAc product inhibition was investigated by carried out the reaction in the presence of GlcNAc intentionally added in various concentrations at the beginning of the reaction. The results showed that the initial concentration of GlcNAc did not inhibit the reaction (**Table 3.2 and Table B9**). However, the product inhibition may be caused by $(GlcNAc)_2$ but the later experiment (**section 8.1**) also ruled out this possibility. Another possible cause for hydrolysis to stop midway maybe that the unhydrolyzed chitin starting material was not in a suitable forms to be hydrolyzed by hevamine, a major endo-chitinase in serum *Hb*.

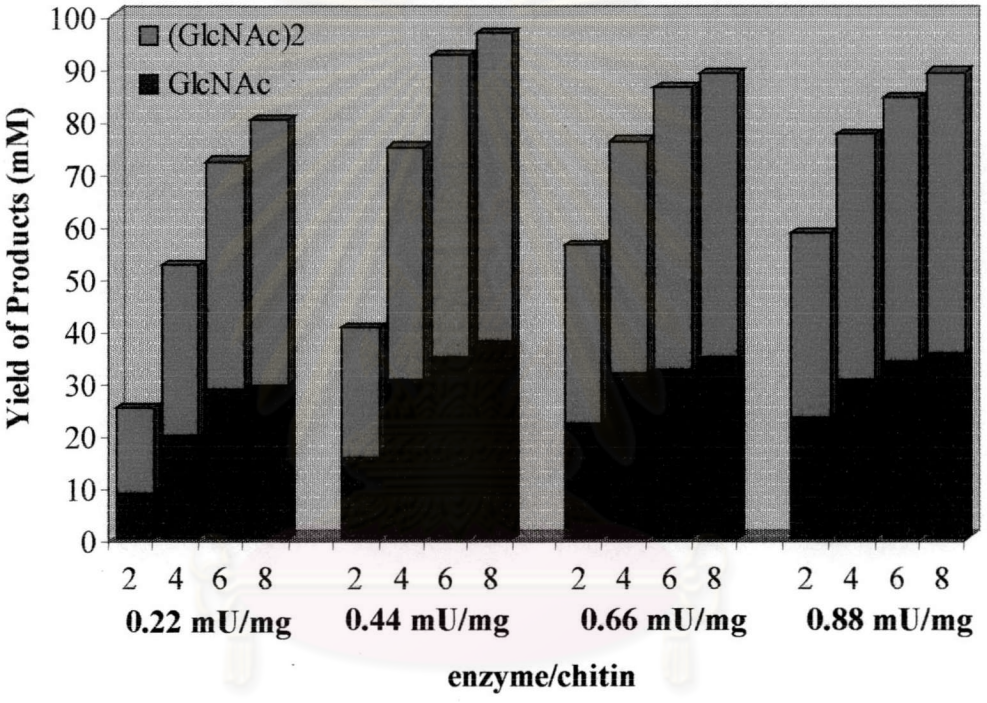


Figure 3.11 The yield of GlcNAc and $(GlcNAc)_2$ with increasing ratio enzyme/chitin study. Condition; [chitin] = 60 mg/mL, enzyme/chitin ratio = 0.22, 0.44, 0.66, 0.88 mU/mg, NaOAc pH 4.0 (0.1 M), 45 °C, 1-8 days.

Table 3.2 The effect of GlcNAc concentration on the product inhibition study.

Initial concentration of GlcNAc (mM)	Yield of products (mM)		Mole ratio of $(GlcNAc)_2$/GlcNAc
	GlcNAc	$(GlcNAc)_2$	
0.00	32.358	44.999	1.4
9.31	31.489	44.439	1.4
18.17	29.931	43.489	1.5
28.21	32.483	46.424	1.4
36.31	33.154	47.258	1.4
47.19	31.032	45.225	1.5

[chitin] = 60 mg/mL, enzyme/chitin ratio = 0.22 mU/mg, NaOAc pH 4.0 (0.1 M), NaN_3 (1000 ppm) at 45 °C on 6th day.

4.9 The stability of serum *Hb*

The serum *Hb* was kept in the refrigerator at 4°C at the pH ~ 3.8. The results from many experiments throughout this research work showed that the enzyme activity was not significantly decreased upon storage for over 10 months providing the similar yields of GlcNAc and $(GlcNAc)_2$ (**Figure 3.12**).

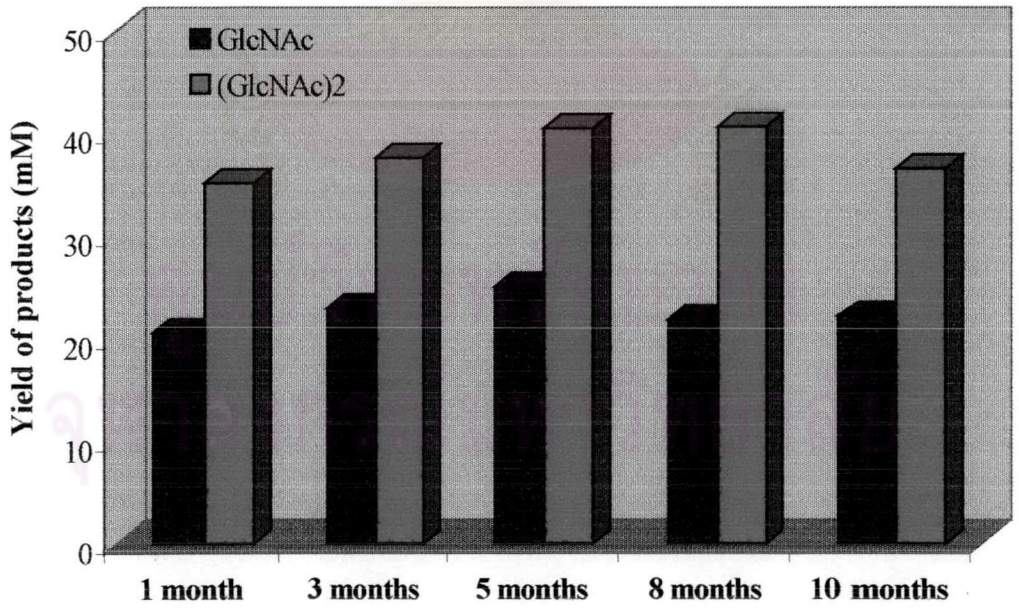


Figure 3.12 The yield of GlcNAc and $(GlcNAc)_2$ in the stability check for serum *Hb*. Condition; [chitin] = 60 mg/mL, enzyme/chitin ratio 0.22 mU/mg NaOAc pH 4.0 (0.1 M), NaN_3 (1000 ppm) at 37 °C, 8th day.

5. Preparative scale preparation of N-acetyl-D-glucosamine and *N,N'*-diacetylchitobiose by using dialysis tubing

K. Matsuoka *et al.* had applied a dialysis technique in the preparation of *N,N'*-diacetylchitobiose by continuous enzymatic degradation of colloidal chitin with chitinase from *Streptomyces griseus*. About 63 g of $(GlcNAc)_2$ (61 %yield, HPLC yield) was produced from 99 g of colloidal chitin. The efficiency of production by this method was 1.2 g $(GlcNAc)_2$ per unit of enzyme.[55] However, the chitinase from *Streptomyces griseus* has a drawback in its product inhibition at low product concentration and its high cost, the maximum concentration of $(GlcNAc)_2$ produced was only 3.3 mg/mL. The results reported in the previous section illustrated that the enzyme in serum *Hb* could produce $(GlcNAc)_2$ with a concentration as high as 50 mM or 21.2 mg/mL. The preparative scale hydrolysis of chitin with the enzyme within dialysis tubing was thus carried out. The use of the dialysis tubing provided two obvious advantages, first it simplified the separation of the products from the enzyme and chitin substrate residue, and second the products could be collected without a necessity to terminate the reaction.

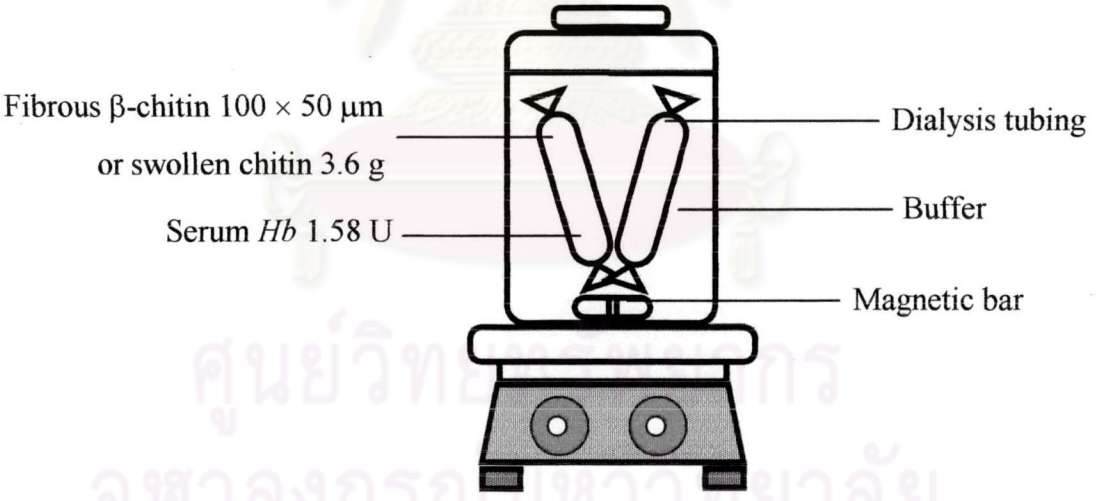


Figure 3.13 Schematic the dialysis tubing **System A**

The reaction was incubated in **System A1** (**Figure 3.13**) that consisted of two closed dialysis bags filled with fibrous β-chitin (3.6 g) and enzyme serum *Hb* (0.79 U) in sodium acetate buffer (0.1 M) pH 4 at 45 °C. The reaction started with a total volume of 810 mL of which 30 mL was inside each of the dialysis bags. The yields of

both GlcNAc and $(GlcNAc)_2$ increased in the first seventh days to 0.9 and 2.3 mM, respectively, and remained relatively constant afterward (**Figure 3.14** and **3.15** and **Table B10**). After 11 days, when the yields of both GlcNAc and $(GlcNAc)_2$ were no longer increased, the external solution (500 mL) of the dialysis tubing was replaced with a freshly prepared buffer (500 mL), and another portion of enzyme (0.79 U) was also added into the dialysis bags. The amounts of both products did not increase proportionally to the increase of the enzyme concentration and only 0.8 mM of GlcNAc and 1.9 mM of $(GlcNAc)_2$ were obtained when the reaction carried out for 10 more days. The HPLC analysis of the combined solution showed that the solution contained GlcNAc (0.22 g, 5.6% yield) and $(GlcNAc)_2$ (1.12 g, 29.8% yield).

The preparative scale hydrolysis of chitin was also performed on another type of substrate preparation. Instead of using fibrous β-chitin, the hydrolysis was carried out by using swollen chitin obtained from a wet grinding of β-chitin by food blender. In this reaction (**System A2**), 1.58 U of the enzyme was used at the beginning without second addition of enzyme. The production profile of this hydrolysate was very similar to that of the **System A1** (GlcNAc = 0.19 g, 4.7% yield) and $(GlcNAc)_2$ = 1.5 g, 28.0% yield).

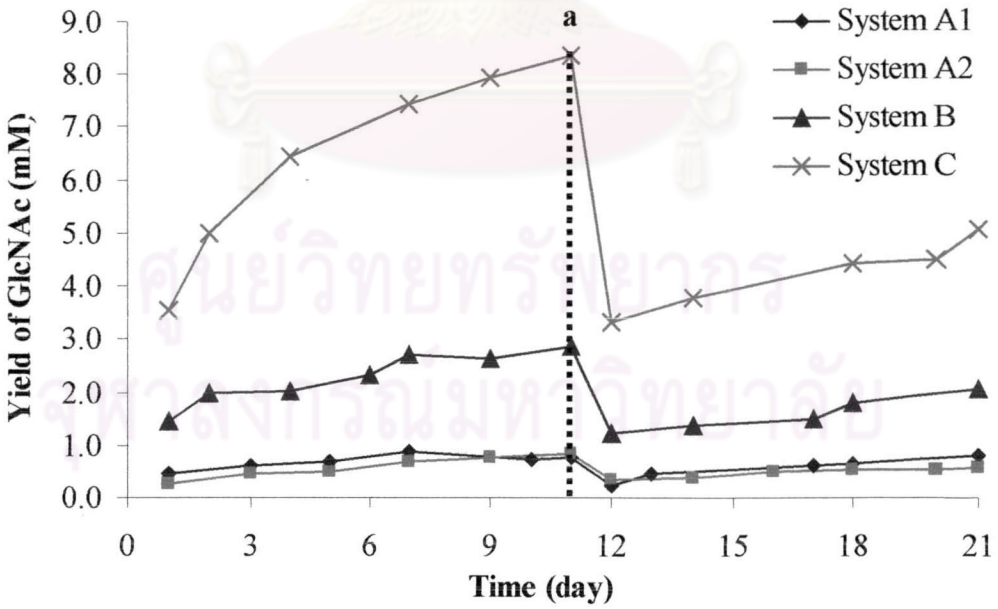


Figure 3.14 Time course of GlcNAc produced by the enzymatic hydrolysis of β-chitin using dialysis **Systems A1**, **A2**, **B**, and **C**. [a]Indicated a replacement of the outer buffer solution of the dialysis bags.

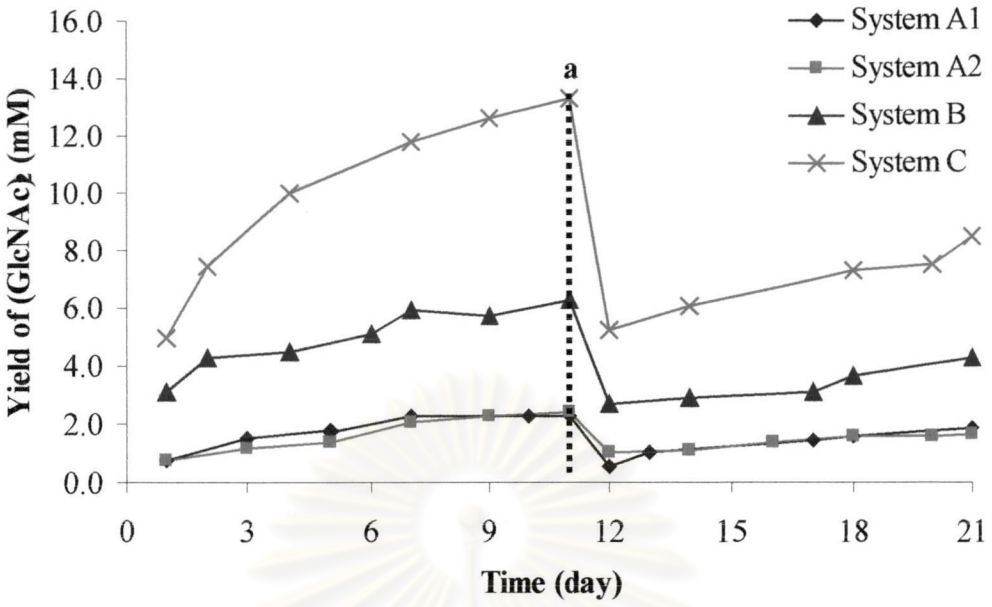


Figure 3.15 Time course of $(GlcNAc)_2$ produced by the enzymatic hydrolysis of β-chitin using dialysis **Systems A1**, **A2**, **B**, and **C**. [a]Indicated a replacement of the outer buffer solution of the dialysis bags.

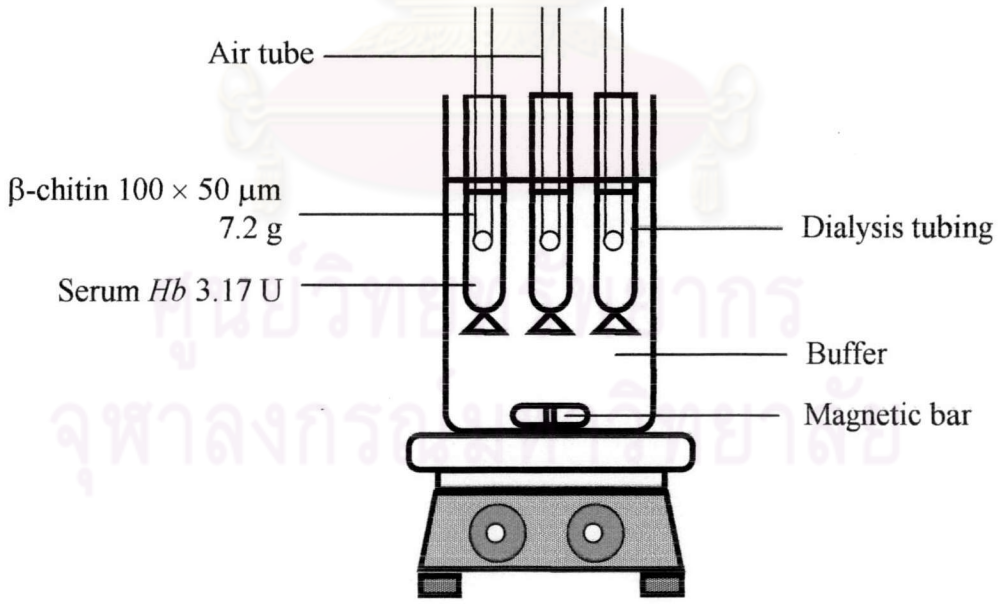


Figure 3.16 Schematic the dialysis tubing **System B**

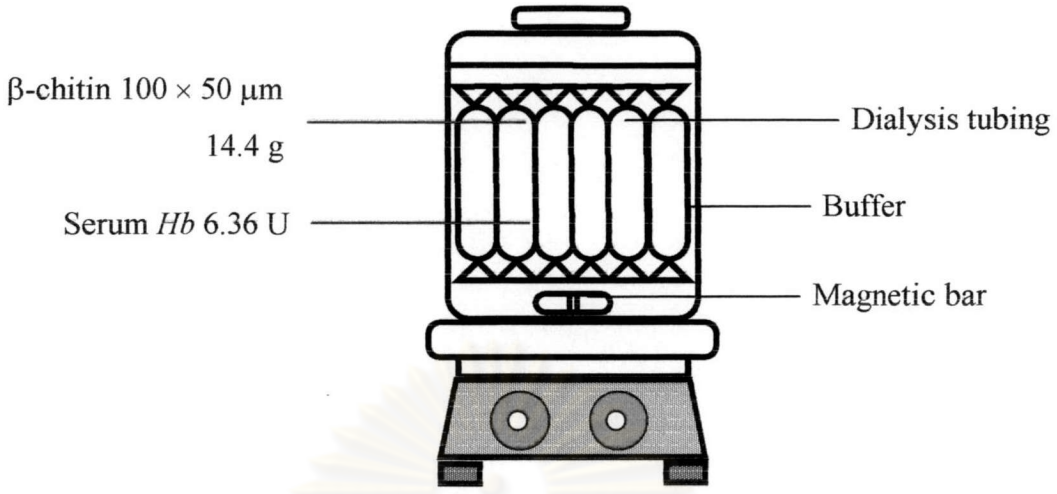


Figure 3.17 Schematic the dialysis tubing **System C**

Both **Systems A1** and **A2** gave low concentration and low conversion of chitin to GlcNAc and $(GlcNAc)_2$ compared to the hydrolysis without dialysis bags. Hence, **System B** and **C** were developed in order to increase production yield and concentration of product (**Figures 3.14** and **3.15**). **System B** consisted of three dialysis bags containing the total chitin of 7.2 g and enzyme of 3.17 U. These three bags were air bubbled continuously throughout the course of the reaction to provide some agitation for the mixture (**Figure 3.16**). While **System C** consisted of six dialysis bags containing the total chitin of 14.4 g and enzyme 6.34 U. No agitation was used in **System C** (**Figure 3.17**). The results clearly showed that the concentration of both GlcNAc and $(GlcNAc)_2$ increased proportionally to the amount of chitin and enzyme used in dialysis bags. The reaction did not seem to require any agitation as system C could produce the product concentration over two times higher than those of system B. Furthermore, the total %yield of GlcNAc and $(GlcNAc)_2$ in system C was also significantly higher than that of system B (**Figure 3.14**, **Figure 3.15**, and **Table 3.3**).

It is also important to emphasize here that the highest concentration of $(GlcNAc)_2$ obtained from **System C** was 13.3 mM (5.7 mg/mL) which was higher than that reported for the hydrolysis with chitinase from *Streptomyces griseus*.[55]

Table 3.3 Total %yield of GlcNAc and $(GlcNAc)_2$ in each various system.

System	β-chitin	Enzyme (U)	Total amount of chitin (g)	Agitation inside the tubes	Total %Yield[c]
A1	100×50 μm	1.58[a]	3.6	Swinging	**37**
A2	Swollen	1.58[b]	3.6	Swinging	**33**
B	50×25 μm	3.17[b]	7.2	Air bubbling	**44**
C	50×25 μm	6.36[b]	14.4	none	**48**

[a]Add further serum *Hb* (0.79U) after replacement the buffer . [b]without added further serum *Hb* after replacement the buffer. [c]HPLC yield.

It is important to note here that although the hydrolysis of fibrous β-chitin with serum *Hb* with the use of dialysis technique in **System C** gave a comparable yield of $(GlcNAc)_2$ to the non-dialysis hydrolysis described in section 4.8, it gave only about half of the enzyme efficiency with longer hydrolysis time (**Table 3.4**). The non-dialysis technique offered shorter hydrolysis time and greater enzyme efficiency that even greater than the best results reported in the literature.[55] The major drawback in using serum *Hb* at the moment is its $(GlcNAc)_2$ yield due to its limit in degree of hydrolysis mentioned earlier.

Table 3.4 $(GlcNAc)_2$ production efficiency from non-dialysis hydrolysis, System C, and literature.[55]

method	chitin	enzyme	Times (day)	$(GlcNA)_2$ yield (%)[a]	Enzyme efficiency (g/U)[b]
Non-dialysis	fibrous β-chitin	serum *Hb*	8	36	1.7
System C	fibrous β-chitin	serum *Hb*	21	37	0.8
literature	colloidal chitin	*Streptomyces griseus*	83	61	1.2

[a]HPLC yield, [b]enzyme efficiency = gram of $(GlcNAc)_2$/unit of enzyme

6. The mechanistic pathways of chitinolysis by enzyme complexes in serum *Hb*

From the data in all previous experiments, the major products from the enzymatic hydrolysis of chitin were $(GlcNAc)_2$ and GlcNAc with a mole ratio of 2:1. In most of enzymatic hydrolysis of chitin, if significant amount of GlcNAc was observed, it would have been a product of hydrolysis of the $(GlcNAc)_2$ by the β-*N*-acetylhexosaminidase activity of the enzyme and the $(GlcNAc)_2$/GlcNAc mole ratio would have dropped dramatically as the reaction proceeded. In the chitinolysis of chitin with serum *Hb*, the $(GlcNAc)_2$/GlcNAc mole ratio was hardly decreased with time (**Figure 3.18**).

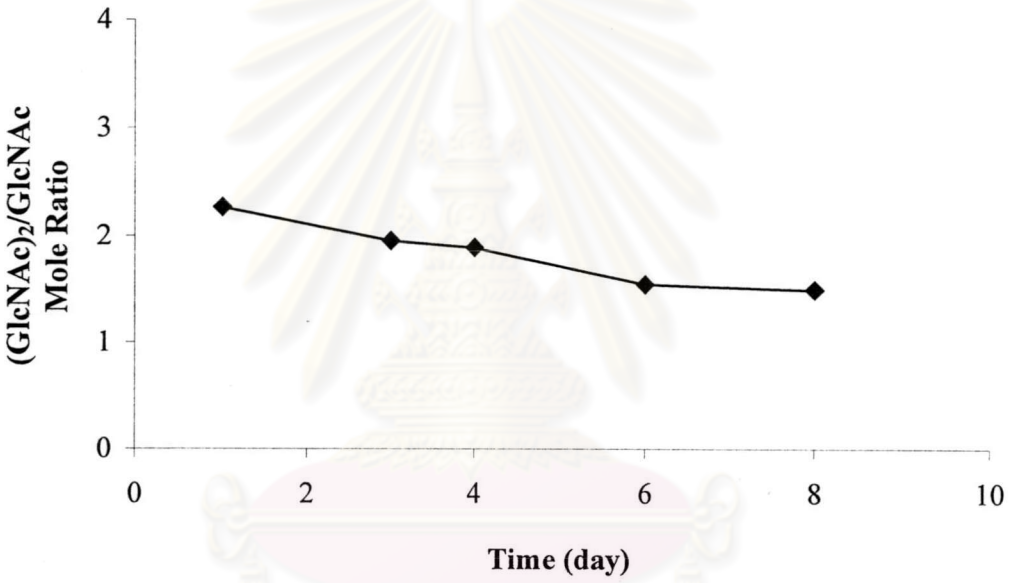


Figure 3.18 The time course of $(GlcNAc)_2$/GlcNAc mole product ratio from the hydrolysis of fibrous β-chitin with serum *Hb*.

The β-*N*- acetylhexosaminidase activity of the enzyme complexs in serum *Hb* was thus evaluated by incubating $(GlcNAc)_2$ with the serum *Hb*. No β-*N*-acetylhexosaminidase activity was observed (**Table 3.5**). With this result, an alternative mechanistic pathway for the derivation of GlcNAc was explored. Further investigation on the substrate specificity was carried out by using $(GlcNAc)_3$ and $(GlcNAc)_4$ as the substrate for the enzymatic hydrolysis.

Table 3.5 The hydrolysis of $(GlcNAc)_2$ with serum *Hb*

Time (hour)	[GlcNAc] (mM)	$[(GlcNAc)_2]$ (mM)
1	Trace	3.574
3	Trace	3.549
5	Trace	3.549
24	Trace	3.578

$[(GlcNAc)_2]$ = 3.585 mM, Serum *Hb* 3.0 mU in acetate buffer pH 4.0 (0.1 M) at 45 °C.

Enzyme from serum *Hb* illustrated hydrolytic activity toward $(GlcNAc)_3$ and gave essentially equal molar of GlcNAc and $(GlcNAc)_2$ as the hydrolytic products (**Table 3.7**). The $(GlcNAc)_4$ was even a better substrate than $(GlcNAc)_3$ as the hydrolysis of $(GlcNAc)_4$ was completed in much shorter time (**Tables 3.6** and **3.7**). The major product from the hydrolysis of $(GlcNAc)_4$ was $(GlcNAc)_2$ and the minor product were GlcNAc and $(GlcNAc)_3$.

Table 3.6 The hydrolysis of $(GlcNAc)_3$ with serum *Hb*

Time (hour)	[GlcNAc] (mM)	$[(GlcNAc)_2]$ (mM)	$[(GlcNAc)_3]$ (mM)
1	0.472	0.559	3.160
3	0.931	1.106	2.525
5	1.921	2.147	1.736
24	3.684	3.521	0.000

$[(GlcNAc)_3]$ = 3.585 mM; Serum *Hb* 3.0 mU in acetate buffer pH 4.0 (0.1 M), at 45 °C.

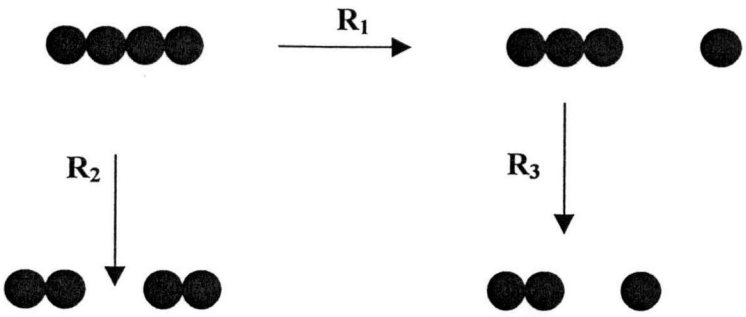
Table 3.7 The hydrolysis of $(GlcNAc)_4$ with serum *Hb*

Time (hrs)	[GlcNAc] (mM)	$[(GlcNAc)_2]$ (mM)	$[(GlcNAc)_3]$ (mM)
1	0.476	3.062	0.419
3	0.929	5.674	0.495
5	1.068	6.295	0.466

$[(GlcNAc)_4]$ = 3.585 mM; Serum *Hb* 3.0 mU in acetate buffer pH 4.0 (0.1 M) at 45 °C.

There have been literatures reported that the major chitinase found produced from para rubber tree is an endo-chitinase belonging to the family 18 glycosylhydrolase called Hevamine. This chitinase hydrolyzes a chitooligosaccharide with molecular size larger than $(GlcNAc)_4$ as it has (-4, -3, -2, -1, +1, +2) binding cleft.[66,93] Serum *Hb* used in this work was thus likely to contain at least another chitinolytic enzyme which can hydrolyze $(GlcNAc)_4$ and $(GlcNAc)_3$.

This enzyme may possess a (-2, -1, +1, +2) binding cleft which can hydrolyze $(GlcNAc)_4$ and $(GlcNAc)_3$ but not $(GlcNAc)_2$. From the relative yields of the hydrolytic products obtained from the hydrolysis of $(GlcNAc)_4$, the difference in binding energy of (-2) site could be estimated as 8.9 kJ/mole (**Scheme 3.1**). A probable hydrolytic pathway in the hydrolysis of chitin by serum *Hb* may involve a hydrolysis of polymeric chitin by the endo-chitinase hevamine to give $(GlcNAc)_5$ as a major product. This $(GlcNAc)_5$ was hydrolyzed by hevamine again to give GlcNAc and $(GlcNAc)_4$ was in turn hydrolyzed by the enzyme just described above to give two molecules of $(GlcNAc)_2$. This mechanism is consistent to the observed $(GlcNAc)_2$/GlcNAc mole ratio of 2. It was suggested from this mechanism that the enzyme hydrolyzing $(GlcNAc)_4$ was a chitobiohydrolase and should be presented in such a small amount that it has never been isolated (**Scheme 3.2**).



$$[GlcNAc] = R_1 + R_3$$
$$[(GlcNAc)_2] = 2R_2 + R_3$$
$$[(GlcNAc)_3] = R_1 - R_3$$

From the hydrolysis of $(GlcNAc)_4$ at 15 mins.

$$0.476 = R_1 + R_3$$
$$3.062 = 2R_2 + R_3$$
$$0.419 = R_1 - R_3$$

Therefore
$$R_1 = 0.448$$
$$R_2 = 1.517$$
$$R_3 = 0.028$$

From $k = Ae^{-Ea/RT}$

$$\ln \frac{k_2}{k_1} = \frac{E_{a1} - E_{a2}}{RT}$$

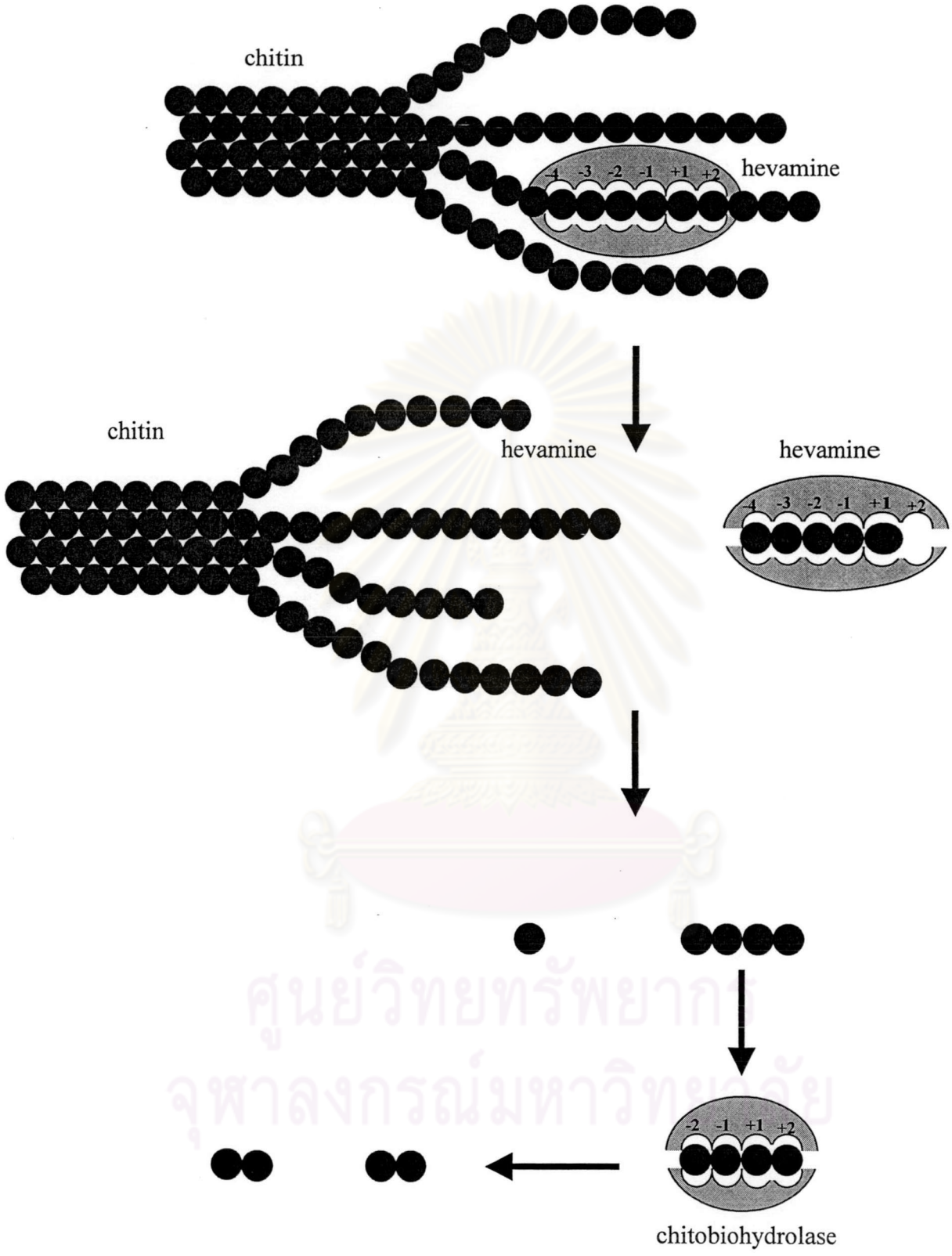
$$\frac{k_2}{k_1} = \frac{R_2}{R_1} = \frac{1.517}{0.448}$$

$$E_{a1} - E_{a2} = 3.39\ RT$$

Binding energy of (-2) site = 8.9 kJ/mole

When; R = 8.314 Joule/mole/K°, T = 318 K°

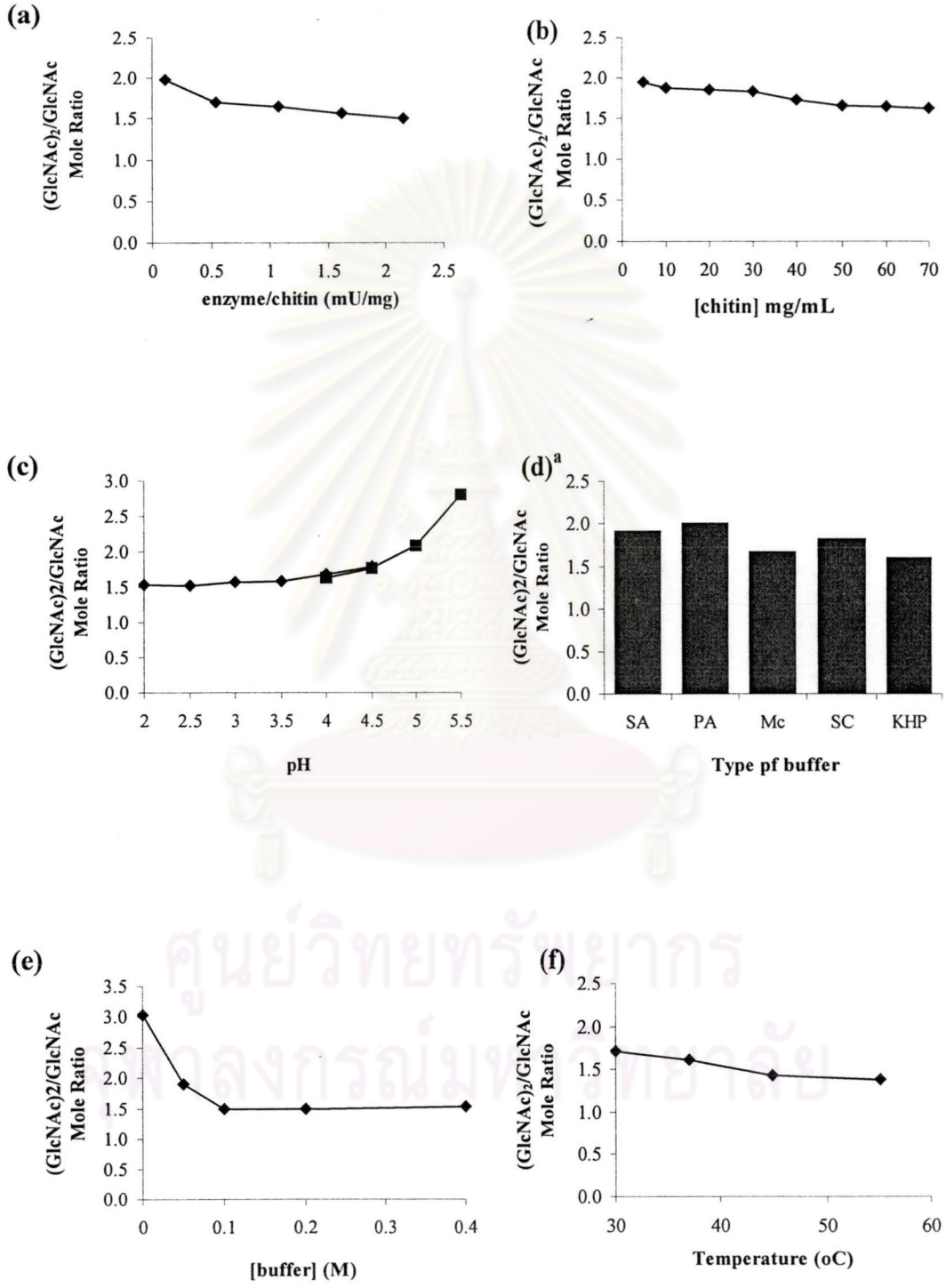
Scheme 3.1 The calculation of binding energy of (-2) site



Scheme 3.2 The mechanistic pathways of chitinolysis by enzyme complexes from serum *Hb*, ● = GlcNAc Unit.

7. The Ratio of GlcNAc and $(GlcNAc)_2$

Noticeably, the $(GlcNAc)_2$/GlcNAc mole ratio of all experiments varied slightly between 1.5-3.0 depending on the conditions (**Figure 3.21**).



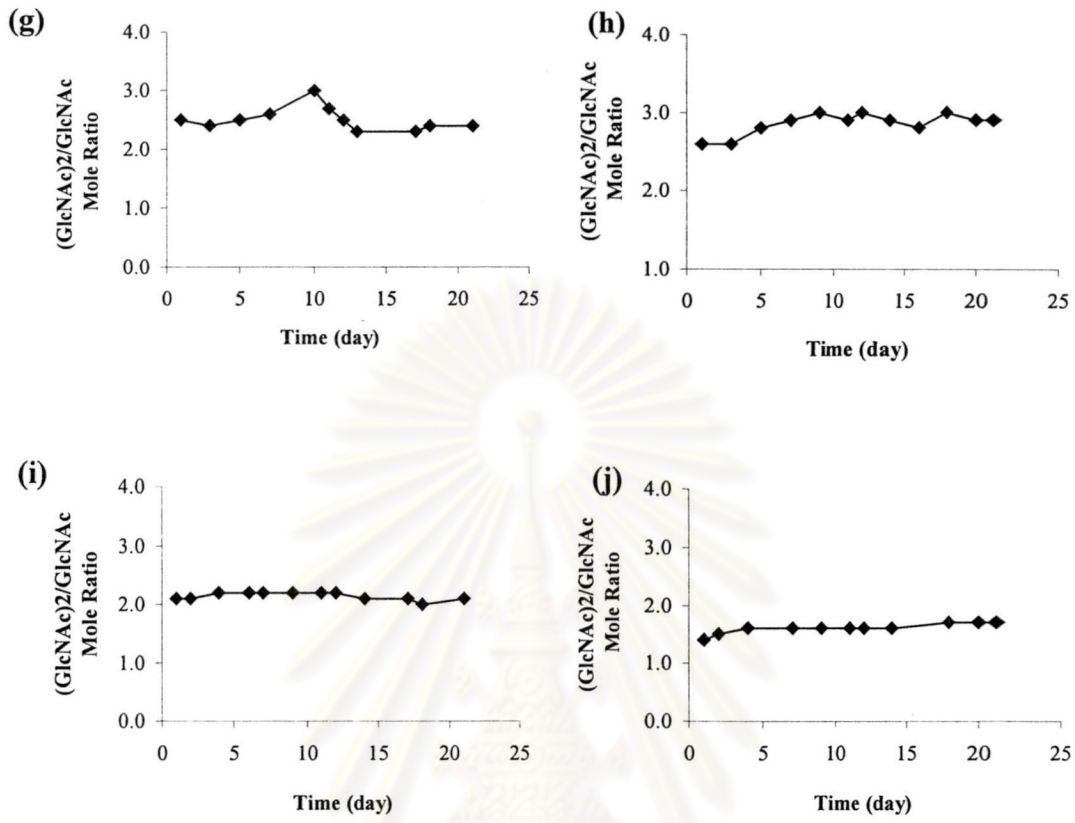


Figure 3.19 The $(GlcNAc)_2/GlcNAc$ mole ratio affected by various condition parameter, (a) enzyme/chitin ratio , (b) chitin concentration, (c) pH, (d) types of buffer,(e) concentration of buffer, (f) temperature, (g) dialysis tubing system A1 (h) dialysis tubing system A2 (i) dialysis tubing system B, (j) dialysis tubing system C.[a] SA = sodium acetate, PA = potassium acetate, Mc = citrate phosphate, SC = sodium citrate, and KHP = potassium hydrogen phthalate.

In stead of plotting the $(GlcNAc)_2$/GlcNAc mole ratio against various parameters as in **Figure 3.19**, only the concentration of $(GlcNAc)_2$ product was set as the X-axis. It is interesting to see that the $(GlcNAc)_2$/GlcNAc mole ratio consistently decreased with increase of product concentration in almost all cases excepted for the case of varying pH (c) (**Figure 3.20**). This observation agrees well with the proposed mechanism.

At lower concentration of the product, chitobiohydrolase may also attack the chain terminus of chitin to produce more $(GlcNAc)_2$ while at higher concentration of product, chitobiohydrolase might have a grater chance to hydrolyze chitooligosaccharides, produced by hevamine, through (-2)(-1)(+1) binding sites that would increase the amount of GlcNAc produced. The significant derivation from this trend observed in the case of varying pH (c) suggested that the hydrolytic pathway might be altered at the high pH. (pH 5.5 in Figure 3.19 graph (c)).

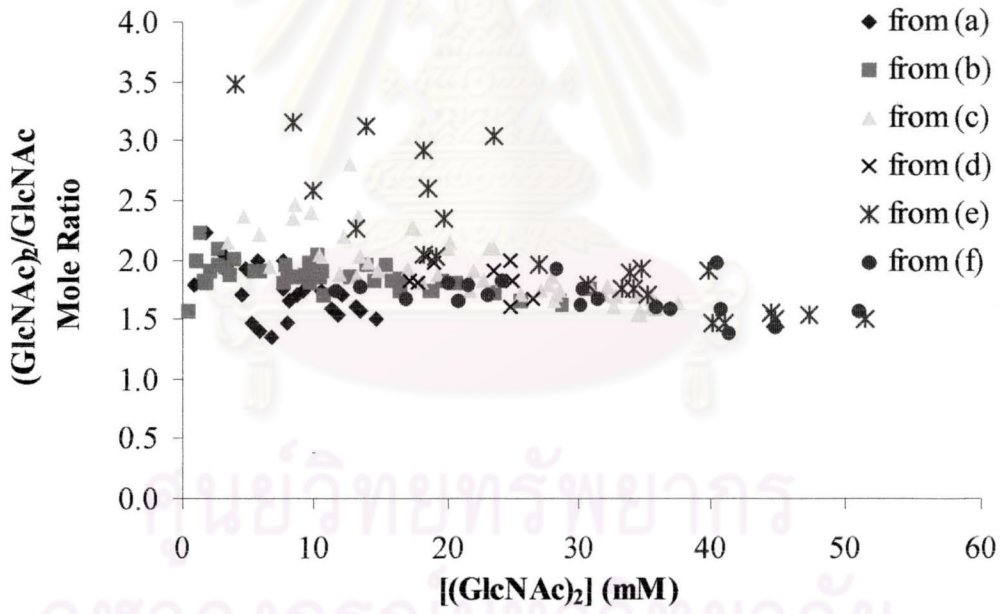


Figure3.20 The relationship between concentration of $(GlcNAc)_2$ and mole ratio $(GlcNAc)_2$/GlcNAc, The data plotted here are the same as graph a-g in **Figure 3.19**.

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

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

The enzyme from serum *Hb* was exhibited reasonably high chitinolytic activity. This enzyme, however, produced two major hydrolytic products, GlcNAc and $(GlcNAc)_2$. There was a method to prepare GlcNAc in one step using an enzyme combination technique.[49] For one-stepped hydrolysis of chitin to give only GlcNAc, the enzyme complexes must contain both chitinase (EC 3.2.1.14) and β-*N*-acetylhexosaminidase (EC 3.2.1.52) activities. The chitinase is responsible for hydrolyzing polymeric chitin chain into smaller chitooligomers, which are in turn further hydrolyzed by β-*N*-acetylhexosaminidase to GlcNAc. Pectinase from *Aspergillus niger* (*An*) was reported to contain high β-*N*-acetylhexosaminidase activity.[49] While serum *Hb* was found in this reserch to contain high chitinase activity but no β-*N*-acetylhexosaminidase activity.

In order to find suitable mixing ratio between pectinase *An* and serum *Hb*. The pectinase *An* was first assayed for its β-*N*-acetylhexosaminidase, specifically, chitobiase, by using $(GlcNAc)_2$ as a substrate. At 1 hour, pectinase *An* (1 mg/mL) hydrolyzed $(GlcNAc)_2$ (5.89 μmole/mL) to produce GlcNAc (2.56 μmole/mL) corresponding to 21.35 mU/mg of chitobiase activity. The chitinase activity of serum *Hb* was 108 mU/mL (see **section 3.2**). The mixing ratio chitobiase activity of pectinase *An* and chitinase activity of serum *Hb* was varied from 0.00 to 3.29.

Most of $(GlcNAc)_2$ was converted to GlcNAc when the mixing ratio between pectinase *An* to serum *Hb* reached 1.65 (106.75 chitobiase U/64.8 chitinase U). At this mixing ratio the GlcNAc/$(GlcNAc)_2$ product mole ratio was already as high as 97:3 (w/w) and further increase in the pectinase *An*/serum *Hb* ratio gave no significant advantage to improve this product ratio (**Figure 3.21** and **Table B14**). By using the enzyme combination of low cost commercial pectinase *An* with serum *Hb*, hydrolysis of β-chitin gave virtually single product as GlcNAc. Purification of GlcNAc from acetate salt, used as a buffer, is however remained as a subject for further investigation.

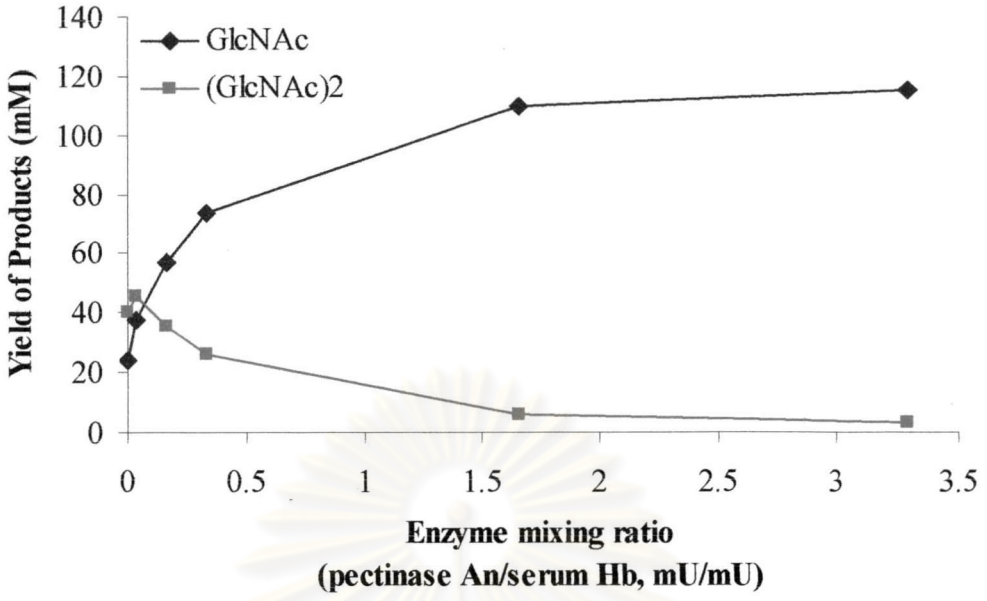


Figure 3.21 The yields of GlcNAc and $(GlcNAc)_2$ obtained by hydrolysis of fibrous β-chitin with various mixing enzyme ratio.

It is also noteworthy to point out here that the hydrolysis of β-chitin by the mixing enzyme also stopped at 50% yield of GlcNAc (**Figure 3.22**) similar to the results observed in the hydrolysis with serum *Hb* solely, described previously. These results suggested that $(GlcNAc)_2$ product inhibition was not a cause for the hydrolysis to stop prematurely. There was thus only one speculation, proposed in section 4.8, left to explain the incompleted hydrolysis of β-chitin with serum *Hb*. The remaining β-chitin substrate left in the reaction was not in the form to be hydrolyzable by hevamine, a major chitinase in serum *Hb*. Such left over substrate might contain GlcNAc units packed in high crystalline domains surrounded by segments of loosen polymeric chain enriched with GlcN units that prevented the attack by hevamine (**Figure 3.23**).

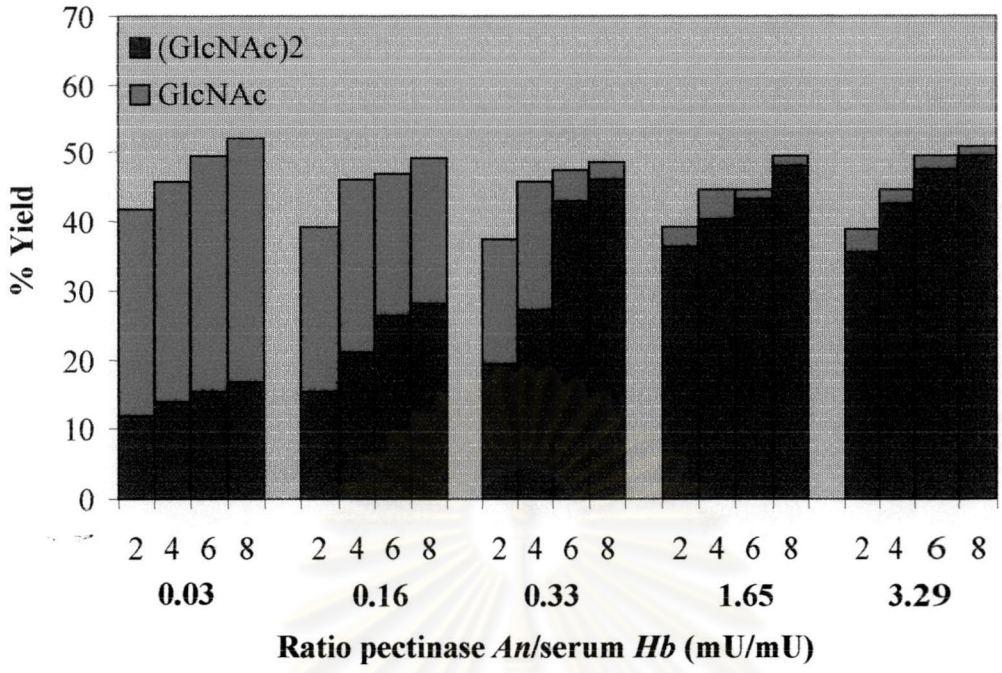


Figure 3.22 The yield of GlcNAc and $(GlcNAc)_2$ obtained by hydrolysis fibrous β-chitin with mixing enzyme at various ratio.

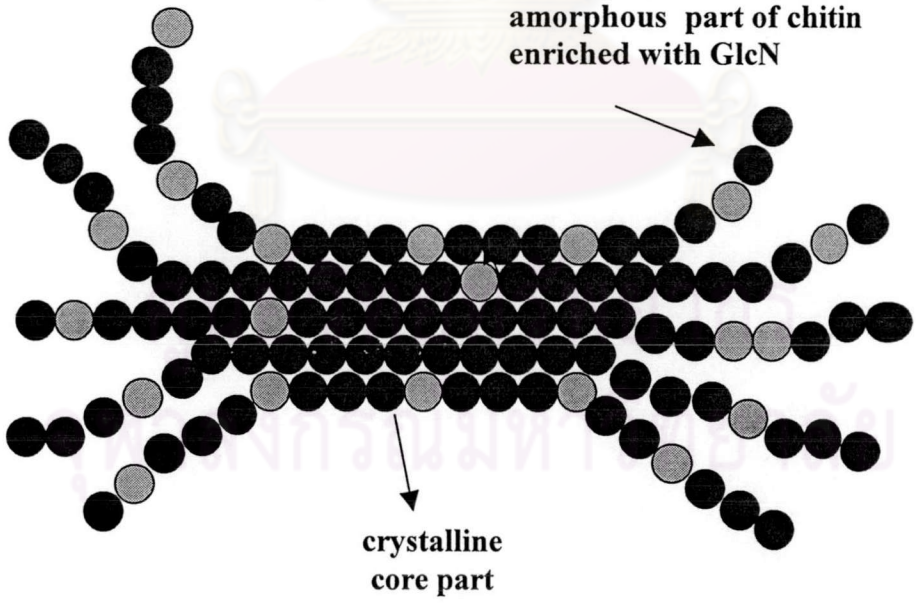


Figure 3.23 The remaining β-chitin substrate left in the reaction, ● GlcNAc and ◍ GlcN.

8.2 Isolation the *N,N'*-diactylchitobiose by gel-filtration chromatography

The hydrolysis of fibrous β-chitin with serum *Hb* gave $(GlcNAc)_2$ as a major product and GlcNAc as a minor product. The GlcNAc can be easily prepared by adding enzyme which has β-*N*-acetylhexosaminidase (EC 3.2.1.52) activity decribed in the last experiment. The $(GlcNAc)_2$, however, needed to be isolated from the crude product by Gel-filtration chromatography (GPC). 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) corresponding to 3.4% GlcNAc and 16.5% $(GlcNAc)_2$ yield. The dried crude product 500 mg was separated by gel-filtration chromatography (**Figure 3.24**).

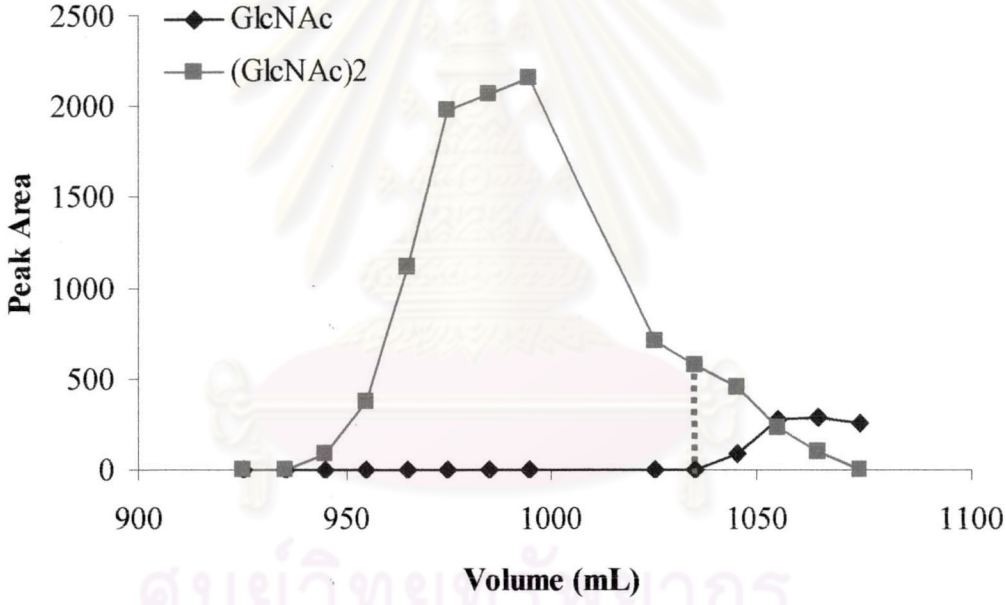


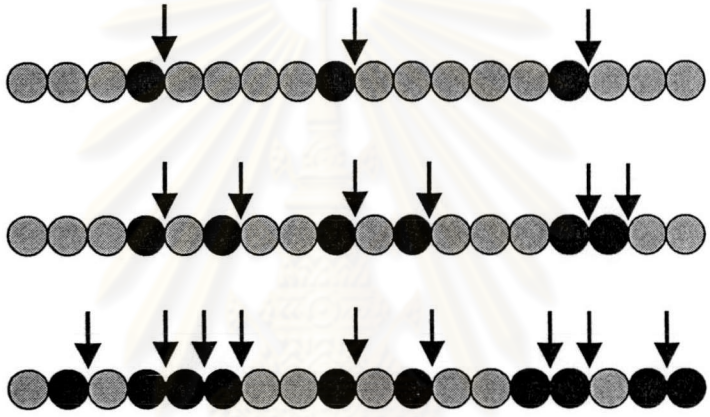
Figure 3.24 The elution profiie of from gel-filtration chromatography.

$(GlcNAc)_2$ started to be eluted out at 940 mL retention volume and exhausted at 1075 mL. GlcNAc began to be eluted out at 1030 mL which overlapped with $(GlcNAc)_2$ elution (**Figure 3.24**). Therefore, the fraction volume from 930 mL to 1040 mL was collected and dried by freeze dryer and the solid $(GlcNAc)_2$ (160 mg) was obtained. The purity of lyophilized $(GlcNAc)_2$ determined by HPLC to be 36% (w/w) corresponding to 92 % recovery. The major impurity was sodium acetate salt

used as a buffer in the reaction. The GlcNAc also was detected as a very minor impurity with less than 0.5% (w/w).

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

The chitooligosaccharides can be easily prepared by enzymatic hydrolysis chitosan with chitinase. S. Aiba reported that chitinase recognizes the GlcNAc residue in chitosan chain.[41] The rates of hydrolysis with chitinase increase with increasing degree of acetylation (DA). The oligomers produced from chitosan with higher DA will generally have lower DP (**Scheme 3.3**).



Scheme 3.3 Speculative representation of the action of chitinase on chitosan having various DA, ● GlcNAc ○ GlcN.

In this works, the digestibility of chitosan by the serum *Hb* was studied. Chitosan from three different sources containing different %DA used (**Table 3.8**)

Table 3.8 %DA and molecular weight of each chitosan.

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]By colloidal titration

[b]By gel permeation chromatography

The hydrolytic time required for the hydrolysis to be completed was studied by viscosity measurements. The substrates (150 mg) were hydrolyzed by the enzyme (3.8 mU) from serum *Hb* at optimum pH and temperature. The viscosity of the hydrolysate was measured at the designated time by Ubbelohde viscometer tube. The viscosity of the hydrolysate dropped sharply in the first 6 hours. After 6 hours, the viscosity of the hydrolysate gradually dropped and became relatively constant after 24 hours (**Figure 3.25** and **Table B15**).

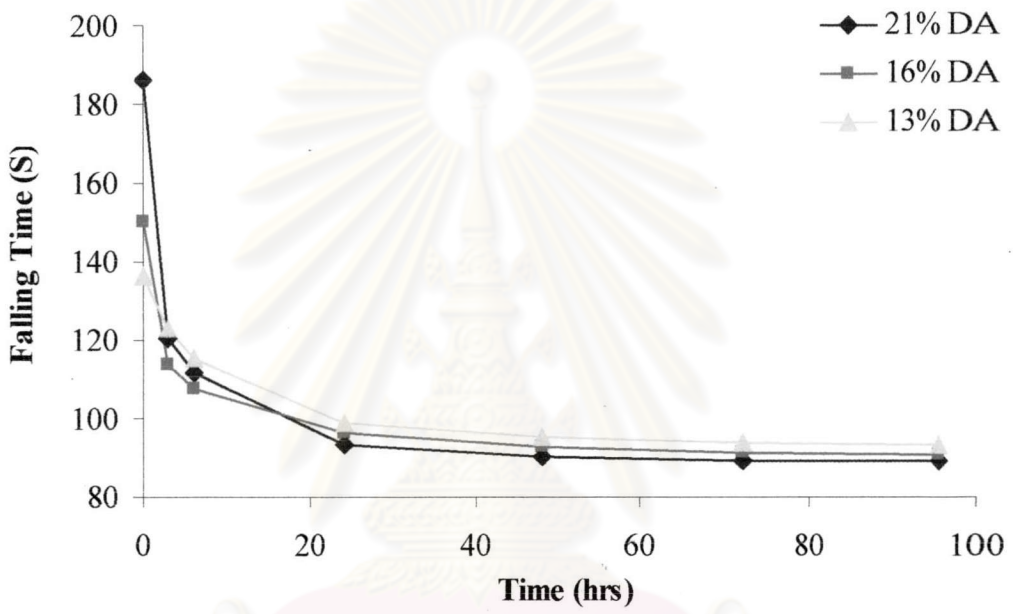


Figure 3.25 Relative viscosity of the hydrolysate as a function of the hydrolysis time.

HPLC was first used as a technique for analyzing the chitooligosaccharide products in the hydrolysate obtained from the hydrolysis of chitosan for 96 hours. In contrast to the literature report for the hydrolysis of chitosan with enzymes from other sources reported by S. Aiba,[41] no chitooligosaccharide smaller than $(GlcNAc)_7$ was detected by HPLC (**Figure B1**). The hydrolysate was thus further analyzed by GPC for their averaged molecular weights.

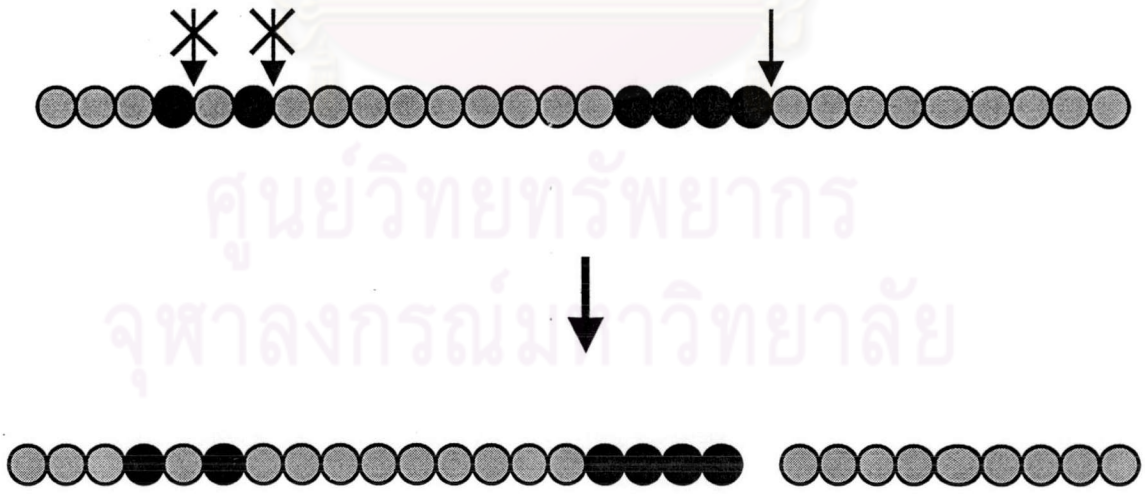
The elution profiles in chromatogram showed that the M_w of chitosan with 13% DA, 16 % DA, and 21 % DA was reduced by 10, 19, and 64 folds, respectively (**Figures B2-B4** and **Table 3.9**).

Table 3.9 The molecular weight of chitooligosaccharide after hydrolysis.[a]

Chitosan source	%DA	$\bar{M}_w$		M_{wI}/M_{wH}
		Initial	Hydrolysis	
Ta-ming	DA 13%	5.1×10^5	5.4×10^4	10
Seafreash	DA 16%	2.8×10^6	1.5×10^5	19
Koyo	DA 21%	6.1×10^6	9.4×10^4	64

[a]Molecular weight was measured by GPC.

The higher %DA, the higher degree of hydrolysis (M_{WI}/M_{WH}) was accomplished. Hevamine, a major chitinase in para serum *Hb*, had been shown to degrade the chitooligomers bigger than $(GlcNAc)_4$. It is possible that hevamine may only recognize a sequence of acetylated glucosamine units of at least 4 units (**Scheme 3.4**). The hydrolysis of chitosan with degree of acetylation 13-21% with serum *Hb* thus gave chitooligosaccharide with molecular sizes larger than $(GlcNAc)_6$. Serum *Hb* was thus suitable for production of low molecular weight chitosan ($M_w \sim 5 \times 10^4 - 1.5 \times 10^5$) However, chitooligosaccharide with low DP may be prepared by using higher %DA of chitosan. Also note that the results observed in this section agreed well with the hydrolytic mechanism proposed in previous sections.



Scheme 3.4 The speculation action of hevamine in chitosan chain, ●GlcNAc, ◯GlcN.