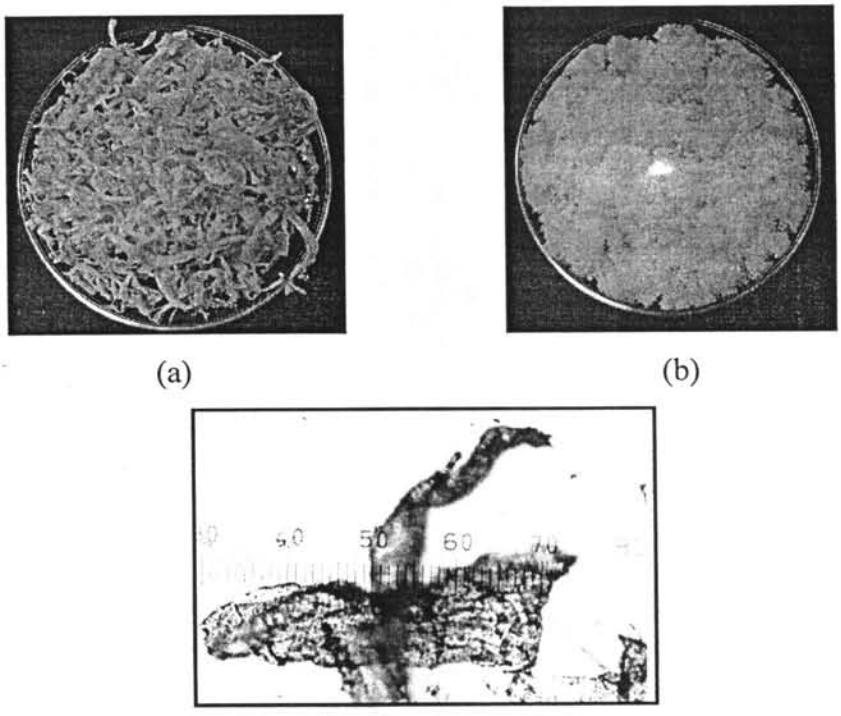
CHAPTER III

RESULTS AND DISCUSSIONS

3.1 Substrates and enzymes

3.1.1 Squid pen chitin (500 μm)

The squid pen chitin was purchased from the industry was in a form of thick fibers with thickness and length in the range of several millimeters and centimeters, respectively (**Figure 3.1 a**). These chitin fibers were cut into millimeter scale in length and milled with an ultracentrifugal mill using a 500 μm sieve. The chitin obtained appeared as a puffy cotton-liked material (**Figure 3.1 b**). The optical microscopy showed that the milled chitin had an average diameter of 50 μm or less and an average length of 0.1-1 mm.



(a) (b)

(c)

Figure 3.1 Photographs of (a) squid pen chitin as purchased, (b) after milling and (c) milled chitin under optical microscope (one minute scale equals to 3 μm)

3.1.2 Colloidal chitin

The coloidal chitin was prepared under acidic condition according to the literature procedure.[67] In the final step, white slurry chitin was obtained. The concentration of colloidal chitin was 33.8 mg/g determined by drying in vacuo. This colloidal chitin was stored at 4 °C for further use in all experiments throughout this thesis.

3.1.3 Chitinase activity assaying

The chitinolytic activities determined for crude enzyme from *Aspergillus fumigatus* fungi and cloned *Serratia marcescens* bacteria, Chi60, were typically 300 mU/mL and 200 mU/mL, respectively, 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 minute.

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

3.2.1 Single batch hydrolysis

Squid pen chitin was hydrolyzed by using the crude enzyme from *Aspergillus fumigatus* at the optimum condition (30% w/v of chitin, enzyme/chitin of 4/1, pH 3 and 40 °C) without buffer to avoid contamination of salt in the desired product. After 2 days, the hydrolysate was boiled, filtered and freeze dried. The sugar content was determined by HPLC. The solid crude product from the hydrolysis of chitin with *A. fumigatus* enzyme contained 60% w/w of GlcNAc and can be translated into 72% yield of GlcNAc from the initial chitin.

Three organic solvents, ethanol, acetone and acetonitrile, were chosen as a precipitating solvents for GlcNAc from its aqueous solution. The aqueous solution of GlcNAc standard (30% w/v) was used as a model studied for the optimum precipitation condition. Ethanol, acetone and acetonitrile were chosen because they are miscible with water. The results showed that ethanol was able to precipitate most of GlcNAc (~80%) from the solution when it was used at five times, or higher, of the water volume. Acetone and acetonitrile precipitated GlcNAc from the solution with significantly lower recovery even at higher organic/water ratio **(Table 3.1)**. Among three solvents tested, ethanol should thus be the solvent of choice for precipitation of GlcNAc. For the subsequent experiments, the precipitation of GlcNAc from crude products was performed by using water-ethanol precipitating system.

Table 3.1 Precipitation of standard GlcNAc in various solvent systems

organic solvent	organic:water	%recovery of precipitate
ethanol	1	54
	2	69
	5	82
	10	75
	15	80
acetone	1	9.6
	2	8.3
	4	54
	8	59
	15	69
acetonitrile	1	0
	2	0
	4	13
	8	19
	15	20

The solution of crude product (30% w/v) was added with ethanol at various ethanol/water ratios to find the optimum ratio which can precipitate GlcNAc from the crude solution with high purity and recovery. Both precipitate and supernatant were analyzed by HPLC for GlcNAc content. The results showed that the amount of precipitate was relatively inconsequential, merely 9-12%, and most of GlcNAc remained in the solution **(Table 3.2)** indicating that the initial GlcNAc concentration was probably too low. The sugar content in the supernatant was also significantly higher than that to the precipitate. With higher ethanol/water ratios, the purity of GlcNAc in the supernatant slightly increased from 72 to 81% (w/w). These results suggested that some impurities with less solubility, under the precipitation condition, are present in the crude product. The higher concentration of GlcNAc in the initial aqueous solution may be necessary to facilitate the precipitation of the sugar.

Table 3.2 Precipitation of 30% (w/v) aqueous crude GlcNAc solution with ethanol

ethanol:water ratio (v/v)	precipitate		supernatant	
	%recovery	%purity	%recovery	%purity
1	12	52	68	72
2	11	66	72	73
5	9	54	73	75
10	12	66	69	77
15	10	45	72	81

3.2.2 Fed-batch hydrolysis

In order to increase the GlcNAc concentration, the fed-batch hydrolyses were performed with the total chitin of 12 g and 25 g and the enzyme (4 U/g of chitin) from *A. Fumigatus*. The hydrolysates were concentrated to the same volume of 30 mL and absolute ethanol was added at the ethanol/water ratio of 7/1. Only the hydrolysate from the batch of 25 g chitin gave significant amont of precipitate. The HPLC analysis of the precipitate showed 90% purity of GlcNAc and %yield of GlcNAc determined from the weight of precipitate was 56%. For the hydrolysis batch starting from 12 g of chitin, the filtrate was dried under vacuum to give 71% yield of GlcNAc with 91% purity (**Table 3.3**). The results suggested that the precipitation of GlcNAc with ethanol can be accomplished from extremely concentrated GlcNAc solution.

Table 3.3 Hydrolysis of chitin by fed-batch method

Total chitin (g)	Total enzyme (U)	GlcNAc obtained from	% yield	%purity
12	48	Filtrate	71	91
25	100	Precipitate	56	90

Since the precipitation method gave the product with light yellow color and 90% purity that is generally unsatisfactory by the standard of chemical reagents. Further improvement in GlcNAc purity was thus performed by decolorization with activated charcoal (0.03 g/1 g GlcNAc). The activated charcoal was stirred with the crude GlcNAc solution (25% w/v) and filtered off. The volume of the filtrate was

reduced to dryness at the temperature lower than 70 °C. The final solid was lightly yellow with GlcNAc purity increasing to 96% (**Table 3.4**). The use of higher activated charcoal/GlcNAc ratio (0.05 g/1 g GlcNAc) increased the purity of GlcNAc to 100% with slightly lower recovery dropping from 51 to 48%. To improve the recovery, the activated charcoal was washed with 10% ethanol (2×25 mL) into the combined filtrate. The result showed that %recovery was improved to 76%. The percent recovery was even higher (92%) with the use of water (3×10 mL) for the activated charcoal washing.

Table 3.4 %purity and %recovery of decolorized GlcNAc

charcoal washing	GlcNAc concentration (% w/v)	charcoal/GlcNAc (w/w)	%purity	%recovery
-	25	0.0283	96	51
-	25	0.0543	100	48
10% EtOH (2×25 mL)	25	0.0623	99	76
water (3×10 mL)	25	0.0507	99	92

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

3.3.1 Hydrolysis of chitin

Squid pen chitin (9 g) was hydrolyzed with the crude enzyme cloned from *Serratia sp.* (9 U) at pH 6 and 37 °C. After 6 days of hydrolysis, and routine work-up, the crude product analyzed by HPLC contained 63% w/w of $(GlcNAc)_2$ and 2% w/w of GlcNAc (8/1 $(GlcNAc)_2$/GlcNAc mole ratio) and gave the calculated yield of $(GlcNAc)_2$ at 43%. This crude product was kept in the solid form after freeze dried before further purification.

3.3.2 Purification of $(GlcNAc)_2$ by precipitation

The same precipitation method was first investigated for the isolation of $(GlcNAc)_2$ from the crude product. Starting from an aqueous solution of the crude product (30% w/v), the precipitation was performed by addition of ethanol at various ethanol/water ratio. The HPLC analysis showed that the purity of $(GlcNAc)_2$ in the

precipitates was 30 – 40% (w/w) dropping from 63% (w/w) in the crude product. The purity of $(GlcNAc)_2$ in the supernatants were comparable to that of the starting crude product (**Figure 3.2**). These result purity of $(GlcNAc)_2$ cannot be improved by precipitation of crude $(GlcNAc)_2$ in ethanol.

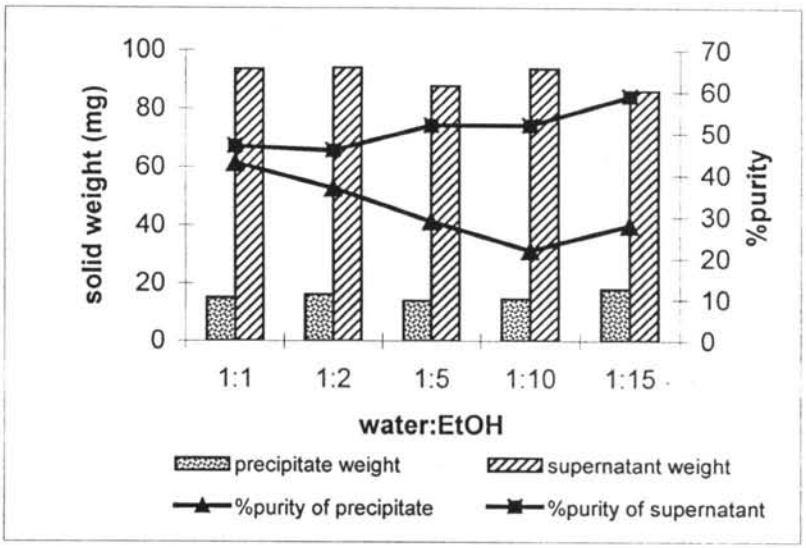


Figure 3.2 %recovery and %purity of precipitates and supernatants from the precipitation of $(GlcNAc)_2$ from crude product.

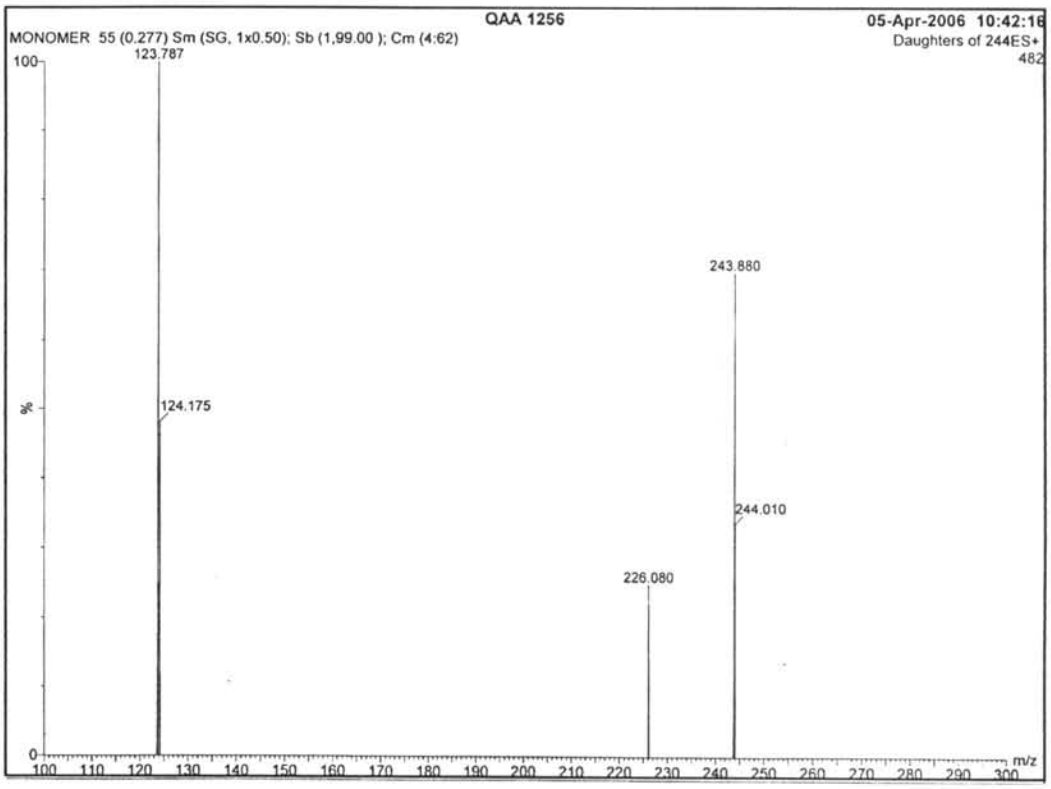
3.3.3 Activated charcoal column chromatography

Recently, Yoon reported that $(GlcNAc)_2$ can be separated from the hydrolysate of chitin hydrolysis by using activated charcoal column to give pure $(GlcNAc)_2$.[66] This method was thus investigated for the isolation of $(GlcNAc)_2$ from the crude product obtained from the hydrolysis in this work. The optimum parameters such as the eluent system, loading capacity of the activated chacoal and volume of crude $(GlcNAc)_2$ solution were determined in this study. Mass spectrometry was used for monitoring the separation between GlcNAc and $(GlcNAc)_2$.

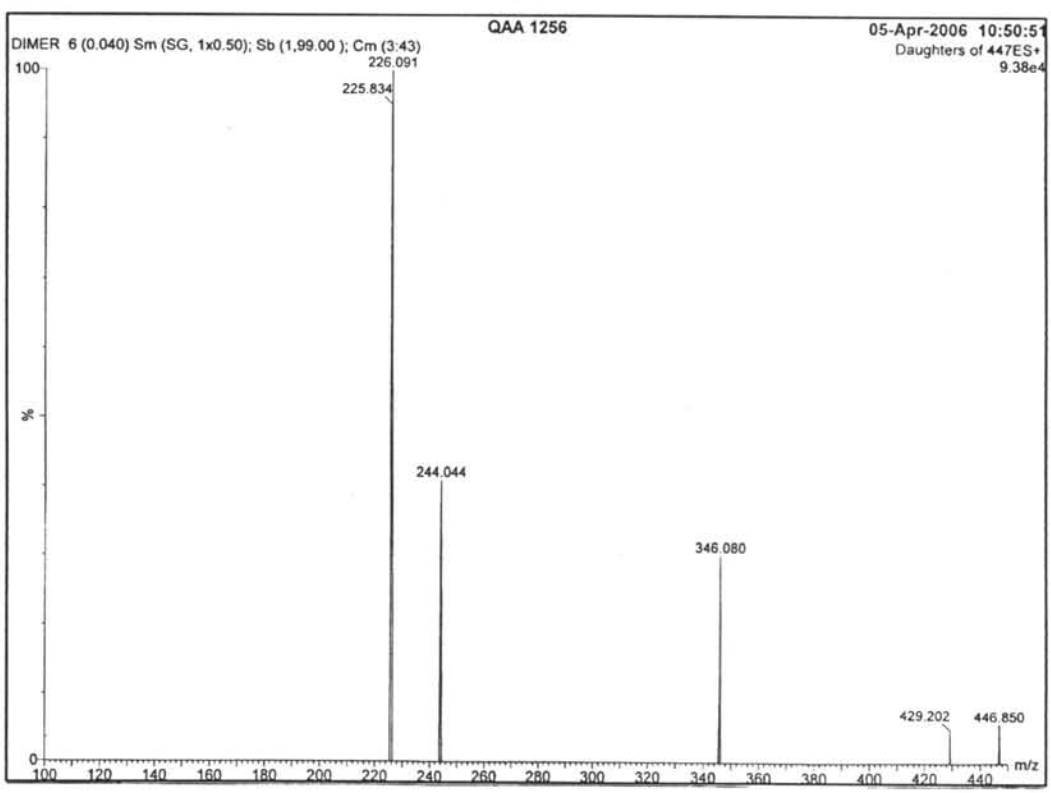
3.3.3.1 Monitoring of separation between GlcNAc and $(GlcNAc)_2$

All parameters in the MS scan mode for standard GlcNAc and $(GlcNAc)_2$ were adjusted to obtained the optimum signals. Voltage at capillary, extractor and RF lens were 40 kV, 3 V and 0 V, respectively. Cone voltage for each standard was 37 and 57 V for GlcNAc and $(GlcNAc)_2$, respectively. Source temperature and desolvation temperature were adjusted to 120 and 350 °C, respectively. The flow rates of N_2 gas were 550 L/hr for desolvation and 50 L/hr for cone. With these parameters

the highest signals of $[GlcNAc+Na]^+$ at m/z of 244 and $[(GlcNAc)_2+Na]^+$ at m/z of 447 were obtained. The resolution of MS1 was adjusted to give peak width between 0.7-0.9 m/z. The low mass (LM) resolution and high mass (HM) resolution were 13.5. The daughter scan mode was selected after the optimum parameter for each standard was obtained. Argon gas was flowed through collision cell. The collision energy was adjusted while the ion energy at the entrance and exit was fixed at 2. The optimum collision energy for fragmentation of GlcNAc and $(GlcNAc)_2$ were 14 and 27 respectively. The daughter ion set of $[AcNH(C_2H_2OH)+Na]^+$ at m/z of 123.787 and $[GlcNAc-H_2O+Na]^+$ at m/z of 226.080 were selected for GlcNAc and the daughter ion set of $[(GlcNAc)-H_2O+Na]^+$ at m/z of 226.091, $[(GlcNAc)_2-(GlcNAc)+Na]^+$ at m/z of 244.044 and $[(GlcNAc)_2-AcNH(C_2H_2OH)+Na]^+$ at m/z of 346.080 were selected for $(GlcNAc)_2$ in the MRM mode. In general, the quantitative determination of GlcNAc and $(GlcNAc)_2$ should be possible by using the peak area of the selected daughter ion from the MRM mode. However the peak areas obtained from the solution of standard GlcNAc or $(GlcNAc)_2$ were not reproducible within 5% probably due to the unstable voltage and the surrounding causing variable degree of ionization. The most reliable quantitative analysis by MS/MS should be performed by using internal isotopic standard. The isotopic standard was not available; therefore, the peak areas of the selected signals have only relative meaning that is sufficient for studying the separation efficiency of the elution. The MS/MS method was chosen in this study also because it has the advantage of being a very rapid and convenient technique



(a)

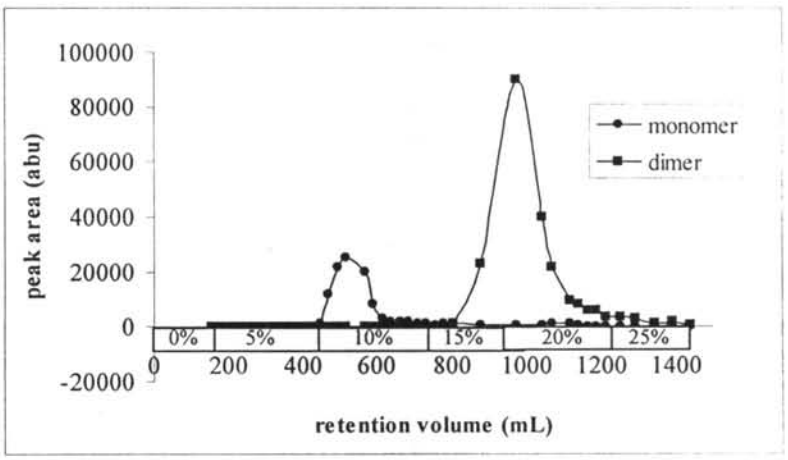


(b)

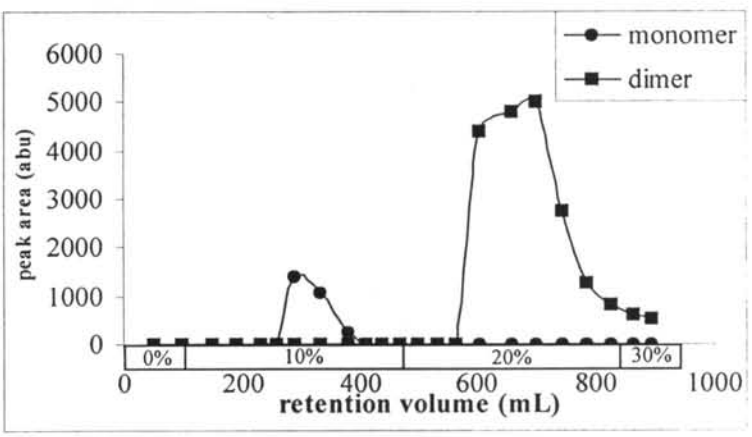
Figure 3.3 Mass spectrum of GlcNAc (a) and $(GlcNAc)_2$ (b)

3.3.3.2 Eluent system

First, the separation of GlcNAc and $(GlcNAc)_2$ was performed by 5% stepwise gradient of water to 30% ethanol. This elution gave very good separation between GlcNAc and $(GlcNAc)_2$ but required about 1400 mL of the eluent and more than 10 hrs to complete the elution of $(GlcNA)_2$ (**Figure 3.4a**). As the chromatogram showed that 10% ethanol eluted only GlcNAc but not $(GlcNAc)_2$, it might be possible to shorten the elution process by using a 10% stepwise gradient elution. The second experiment, 0-10% ethanol was used for the first 500 mL elution volume, where the elution of GlcNAc was completed (**Figure 3.4b**). After the elution of GlcNAc was completed, 20% ethanol was used to elute $(GlcNAc)_2$. . In this second experiment, the elution was terminated after 8 hr where the most of the $(GlcNAc)_2$ was eluted with the total elution volume of about 1000 mL. These results suggested that the 10% stepwise gradient elution was preferred for the faster elution.



(a)



(b)

Figure 3.4 Chromatogram of GlcNAc and $(GlcNAc)_2$ eluted by 5% ethanol stepwise gradient (a) and 10% ethanol stepwise gradient (b)

3.3.3.3 Loading capacity of the activated charcoal column

Various amounts of the crude product from the enzymatic hydrolysis were eluted through a column containing 60 g of the activated charcoal. The chromatograms showed that even with 1.86 g of total sugars, a baseline separation (R = 1.7) between GlcNAc and $(GlcNAc)_2$ was still observed (**Figure 3.5 and Table 3.5**). After freeze drying of the combined fractions containing $(GlcNAc)_2$, the sugar was obtained as a white solid with high purity.

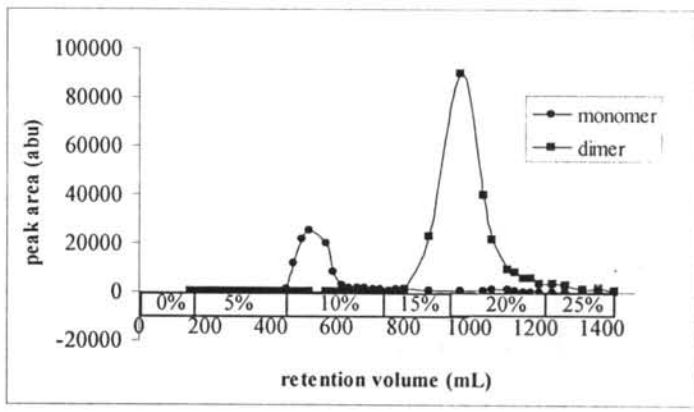


Figure 3.5 Chromatogram of activated charcoal column loading by 0.44 g of total GlcNAc and $(GlcNAc)_2$.

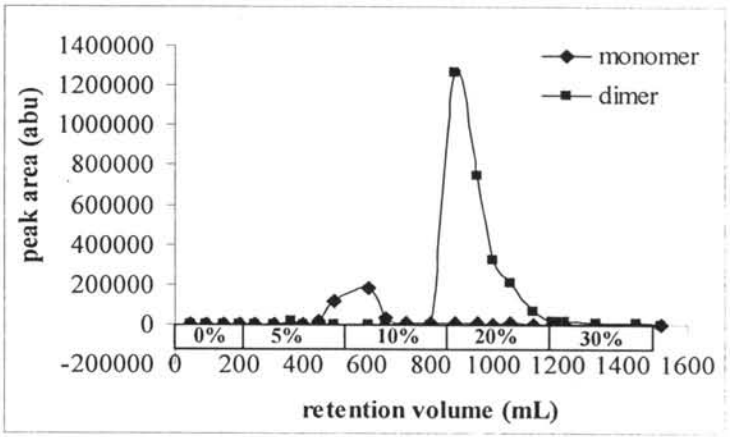
Table 3.5 Column resolution between GlcNAc and $(GlcNAc)_2$ at various amount of loading sugars

GlcNAc+$(GlcNAc)_2$ Weight (g)	$(GlcNAc)_2$ obtained (g)	R	% purity determined by HPLC
0.44	0.37	1.8	98
0.89	0.85	3.2	100
1.86	1.63	1.7	90

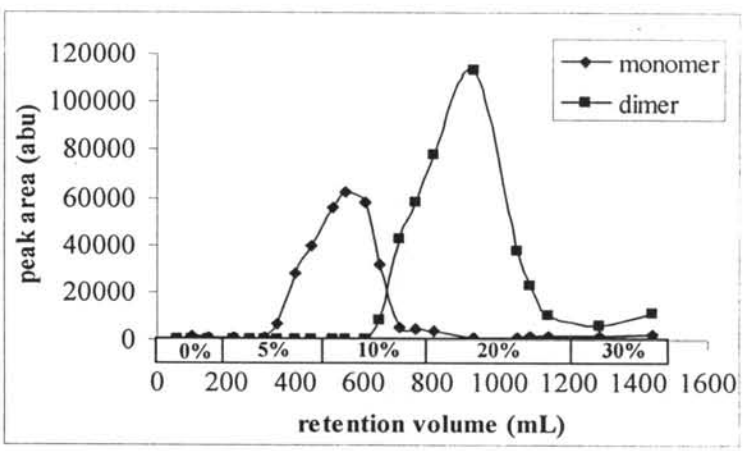
3.3.3.4 Effect of the loading volume of the sugar solution

In the preparation of $(GlcNAc)_2$ in large scale, the freeze drying of the hydrolysate is a very time and energy consuming step. To investigate for the possibility to omit this step, the hydrolysate (~150, 300 mL) from the enzymatic hydrolysis was directly loaded into the activated charcoal column. The chromatograms showed that the separation of GlcNAc and $(GlcNAc)_2$ in the hydrolysate was possible without the freeze drying step (**Figure 3.6**). The sugar loading was also increased over 2 g for further determination of the column capacity.

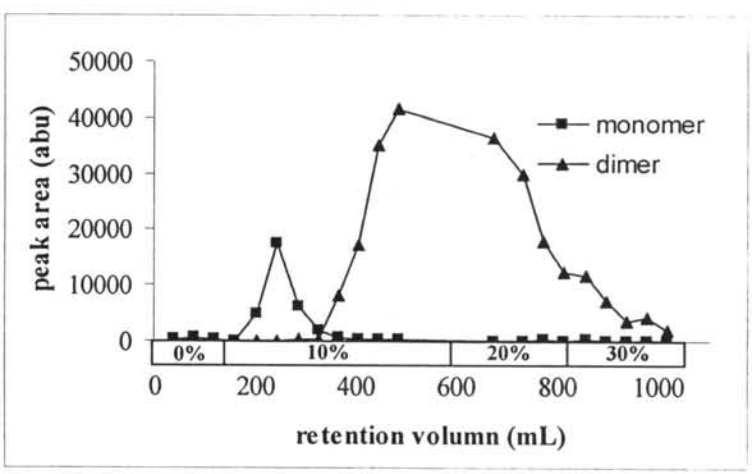
The results showed that the sugar loading can be increased up to 2.58 g with a little overlapping near the baseline.



(a)



(b)



(b)

Figure 3.6 Chromatograms of GlcNAc and $(GlcNAc)_2$ starting from diluted hydrolysate (250 mL) with starting sugars of 1.86 g/30 mL (a), 2.27 g/150 mL (b) and 2.58 g/300 mL (c)

3.3.3.5 Effect of enzyme/chitin ratio to the product yield

For increasing the product yield of $(GlcNAc)_2$ chitin was hydrolyzed by using enzyme 5 U/1 g of chitin in the total reaction volume of 300 mL. After hydrolysis for 6 days the hydrolysate (150 mL from total 250 mL) was loading into the activated charcoal column. The result showed that $(GlcNAc)_2$ (2.07 g) was produced from 150 mL of hydrolysate which can be calculated to 40% yield with 100% purity.

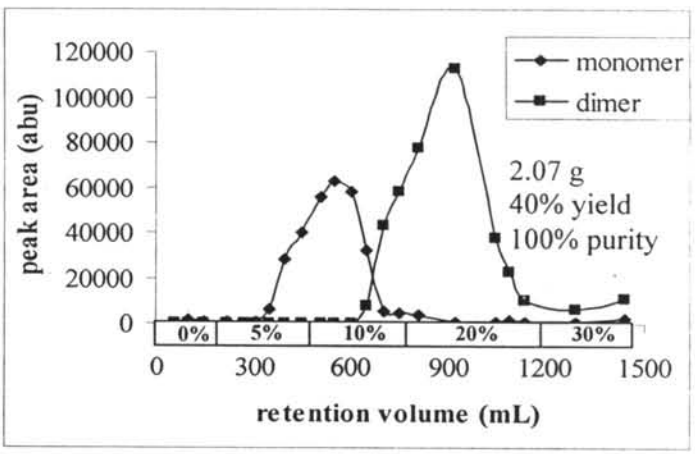


Figure 3.7 Chromatogram of GlcNAc and $(GlcNAc)_2$ from the hydrolysis of chitin by enzyme 5 U/1 g of chitin.

3.4 Purity analysis of sugars

The purities of isolated GlcNAc and $(GlcNAc)_2$ were determined by using two different techniques; high performance liquid chromatography (HPLC) and nuclear magnetic resonance (^{1}H NMR). In the HPLC technique, the amount of the sugar was determined against the calibration line of the corresponding standard sugar and the %purity was calculated according to the following equation.

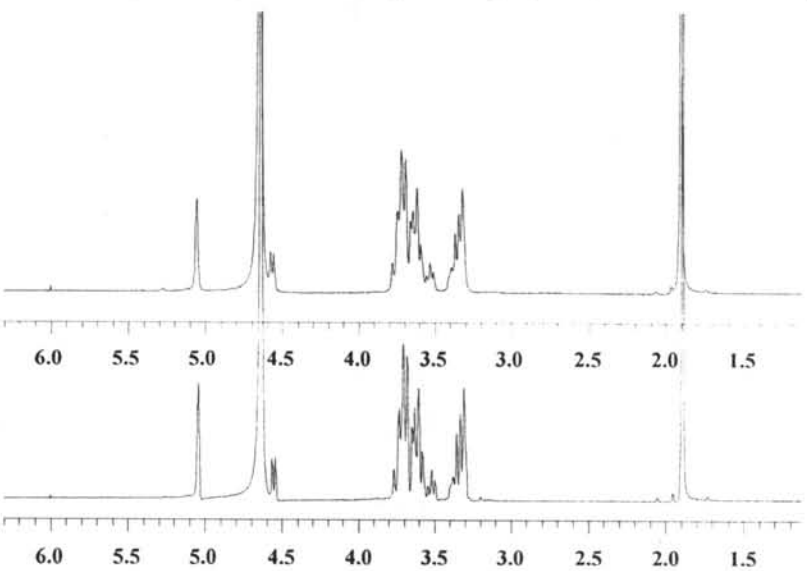
$$\% \text{ purity} = \frac{\text{HPLC weight} \times 100}{\text{sample weight}}$$

The analysis showed that GlcNAc isolated by precipitation with ethanol from very concentrated solution had 90% purity. The purity could be improved to 100% by decolorization with activated charcoal with some product loss. The isolation of $(GlcNAc)_2$ by activated charcoal column with suitable loading weight of sugar and eluent system gave the product with 100% purity (**Table 3.6**).

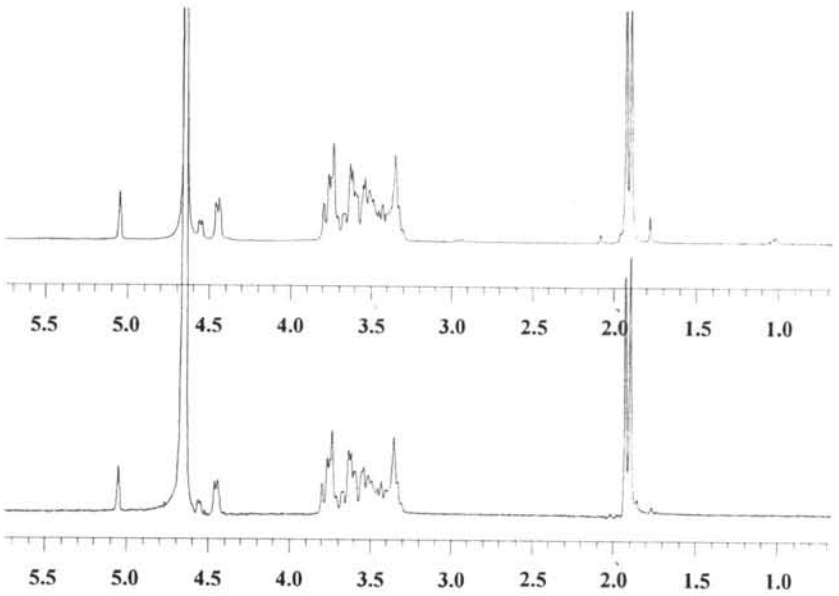
Table 3.6 %purity and %isolated yield of purified products by using different purification method

Method of purification	product	%purity	% isolated yield
Precipitation	GlcNAc	90	71
Precipitation + decolorization	GlcNAc	99	64
Activated charcoal column	$(GlcNAc)_2$	100	40

The determination of purity by using ^1H NMR technique was performed by comparing the ^1H NMR spectrum of the isolated product to the spectrum of the corresponding standard sugar. The spectrum of GlcNAc obtained from the precipitation followed with decolorization was identical to that of the standard GlcNAc confirming high purity of the isolated product (**Figure 3.7 a**). In the case of $(GlcNAc)_2$ isolated by the activated charcoal column chromatography, three minor signals around 1.0, 1.8, 2.1 and 3.0 ppm which could not be identified were observed. With the exception of these three minor signals, all other signals were identical to those of the standard $(GlcNAc)_2$ confirming the high purity of the isolated product.



(a)



(b)

Figure 3.7 NMR spectrum of standard GlcNAc and precipitated GlcNAc (a) and standard $(GlcNAc)_2$ and $(GlcNAc)_2$ purified by activated charcoal (b)