REFERENCES

1. Kramer, K., and Koga, D., "Insect Chitin: Physical State, Synthesis, Degradation and Metabolic Regulation", *Insect Biochem.*, **1986**, *16*, 851-877.
2. Tracey, M. V., "Chitin", *Rev. Pure Appl. Chem.*, **1957**, *7*, 1-14.
3. Blackwell, J., "Physical Methods for the Determination of Chitin Structure and Conformation", *Method Enzymol*, **1988**, *161*, 435-442.
4. Minke, R., and Blackwell, J., "The Structure of α-Chitin", *J. Molec. Biol.*, **1987**, *120*, 167-181.
5. Goosen, M. F. A., *Application of Chitin and Chitosan*, Pennsylvania: Technomic Publishing, **1997**, pp 79-86.
6. Shahidi, F., and Synowiecki, J., "Isolation and Characterization of Nutrients and Value-Added Products from Snow Crab (*Chinoeceles opilio*) and Shrimp (*Pandalus borealis*) Processing Discards", *J. Agric. Food. Chem.*, **1991**, *39*, 1527-1532.
7. Carroad, P. A., and Tom, R. A., "Bioconversion of The Chitin Wastes: Process Conception and Selection of Microorganism", *J. Food Sci.*, **1978**, *43*, 1158-1161.
8. Muzzarelli, R. A. A.; Jeuniaux, C., and Gooday, G. W., *Chitin in Nature and Technology*, New York: Plenum Press, **1986**, 287-293.
9. Shahidi, F.; Kamil, J.; Arachchi, V., and Jeon, Y. J., "Food Application of Chitin and Chitosans", *Trends in food science & Technology*, **1999**, *10*, 37-51.
10. Akiyama, K.; Kawazu, K., and Kobayashi, A., "A novel Method for Chemo-Enzymatic Synthesis of Elicitor-Active Chitosan Oligomers and Partially *N*-Deacetylated Chitin Oligomers Using *N*-Acetylated Chitotrioses as Substrates in A Lysozyme-Catalyzed Transglycosylation Reaction System", *Carbohyd. Res.*, **1995**, 279, 151.
11. Brodelius, P.; Funk, C.; Haner, A., and Villegas, M., "A Procedure for The Determination of Optimal Chitosan Concentrations For Elicitation of Cultured Plant Cells", *Phytochemistry*, **1989**, *28*, 2651-2654.

12. Yamada, A.; Shibuya, N.; Kodama, O., and Akatsuka, T., "Induction of Phytoalexin Formation in Suspension-Cultured Rice Cells by *N*-Acetyl-chitooligosaccharides", *Biosci. Biotech. Biochem.*, **1993**, *57*, 405-409.

13. Vander, P.; Vrum, K. M.; Domard, A.; Eddine, N.; Gueddari, E., and Moerschbacher, B. M., "Comparison of The Ability of Partially *N*-Acetylated Chitosans and Chitooligosaccharides to Elicit Resistance Reactions in Wheat Leaves", *Plant Physiol.*, **1998**, *118*, 1353-1359.

14. Suzuki, S., "Studies on Biological Effects of Water Soluble Lower Homologous Oligosaccharides of Chitin and Chitosan", *Fragrance J.*, **1996**, *15*, 61-68.

15. Suzuki, K.; Tokoro, A.; Okawa, Y.; Suzuki, S., and Suzuki, M., "Enhancing Effects of *N*-Acetylchitooligosaccharide on The Active Oxygen-Generating and Microbicidal Activity of Peritoneal Exudate Cells in Mice", *Chem. Pharma. Bull.*, **1985**, *33*, 886-888.

16. Tokoro, A.; Tatewaki, N.; Suzuki, K.; Mikami, T.; Suzuki, S., and Suzuki, M., "Growth-Inhibitory Effect on Hexa-*N*-Acetylchitohexoase and Chitohexaose Against Meth-A Solid Tumor", *Chem. Pharma. Bull.*, **1988**, *36*, 784-790.

17. Suzuki, K.; Tokoro, A.; Okawa, Y.; Suzuki, S., and Suzuki, M., "Effect of *N* - Acetylchitooligosaccharides on Activation of Phagocytes",*Microbiol. Immunol.*, **1986**, *30*, 777-787.

18. Tokoro, A.; Kobayashi, M.; Tatewaki, N.; Suzuki, K.; Okawa, Y.; Mikami, T.; Suzuki, S., and Suzuki, M., "Protective Effect of *N*-Acetyl Chitohexaose on *Listeria monocytogens* Infection in Mice", *Microbiol. Immunol.*, **1989**, *33*, 357.

19. Tsukada, K.; Matsumoto, T.; Aizawa, K.; Tokoro, A.; Naruse, R.; Suzuki, S, and Suzuki, M., "Antimetastatic and Growth Inhibitory Effect of *N*-Acetylchitohexaose in Mice Bearing Lewis Lung Carcinoma", *Jpn. J. Cancer, Res.*, **1990**, *81*, 259-265.

20. Suzuki, K.; Mikami, T.; Okawa, Y.; Suzuki, S., and Suzuki, M., "Antitumor Effect of Hexa-*N*-Acetylchitohexaose and Chitohexaose", *Carbohydr. Res.*, **1986**, *151*, 403-408.

21. Shikhman, A.R.; Kuhn, K.; Alaaeddine, N., and Lotz, M., "*N*-Acetylglucosamine Prevents IL-1 Beta-Mediated Activation of Human Chondrocytes", *J. Immunology*, **2001**, *166*, 5155-5160.

22. Hatano, K.; Miyakuni, Y.; Hayashida, K.; and Nakagawa, S., "Effects and Safety of Soymilk Beverage Containing *N*-acetyl glucosamine on Osteoarthritis", *Japanese Phrmacology and Therapeutics*, **2006**, *34(1)*, 149-165.

23. Hiroshi, H., "Disaccharide as Starting Materials for The Synthesis of Valuable Compounds", *Trends in Glycoscience and Glycotechnology*, **2000**, *12*, 185-190.

24. Takahashi, S.; Terayama, H.; Koshino, H., and Kuzuhara, H., "Synthesis of Novel Azapseudodisaccharide Related to Allosamidin Employing *N,N'*-Diacetylchitobiose as A Key Material", *Tetrahedron*, **1999**, *55*, 14871-14884.

25. Nishimura, S. and Kuzuhara, H., "Synthesis of a Peripheral Trisaccharide Sequence of lutropin, a pituitary glycoprotein hormone; use of chitobiose as a key starting material", *Carbohydr. Res.*, **1990**, *206(2)*, 207-217.

26. Kobayashi, K.; Tawada, E.; Akaike, T. and Usui, T., "Artificial glycopolypeptide conjugates: simple synthesis of lactose- and *N,N'*-diacetylchitobiose-substituted poly(L-glutamic acid)s through *N*-β-glycoside linkages and their interaction with lectin", *Biochimica et Biophysica Acta*, **1997**, *1336*, 117-122.

27. Rupley, S. "The Hydrolysis of Chitin by Concentrated Hydrochloric Acid, and The Preparation of Low-Molecular-Weight Substrates For Lysozyme A", *Biochim. Biophys. Acta*, **1964**, *83*, 245-255.

28. Takahashi, Y.; Miki, F., and Nagase, K., "Effect of Sonolysis on Acid Degradation of Chitin to Form Oligosaccharides, *Bull. Chem. Soc. Jpn.*, **1995**, *68*, 1851-1857.

29. Bosso, C.; Defaye, J.; Domard, A.; Gadelle, A., and Pedersen, C., "The Behavior of Chitin Towards Anhydrous Hydrogen Fluoride. Preparation of β-(1→4)-Linked 2-Acetamido-2-Deoxy-D-Glucopyranosyl Oligosaccharides", *Carbohyd. Res.*, **1986**, 156, 57-68.

30. Horowits, S.; Roseman, S., and Blumemthal, H. J., "The Preparation of Glucosamine Oligosaccharides. I. Separation", *J. Am. Chem. Soc.*, **1957**, *79*, 5046.

31. Inaba, T.; Ohguchi, T.; Iga, Y., and Hasegawa, E., "Synthesis of 4-Methylcoumarin-7-yloxy Tetra-N-Acetyl-β-Chitotetraoside, A Novel

Synthetic Substrate for The Fluorometric Assay of Lysozyme", *Chem. Pharm. Bull.*, **1984**, *32*, 1597-1603.

32. Kurita, K.; Tomita, K.; Ishii, S.; Nishimura, S., and Shimoda, K., "Beta-Chitin as A Convenient Starting Material for Acetolysis for Efficient Preparation of *N*-Acetylchitooligosaccharides", *J. Poly. Sci.: Part A: Poly. Chem.*, **1993**, *31*, 2393-2395.
33. Skjak-Braek, G.; Anthonsen, T., and Sanford, P., *Chitin and Chitosan*, London: Elsevier, **1989**, pp 415-429.
34. Nordtveit, R. J.; Varum, K. M., and Smidsrod, O., "Degradation of Fully Water-Soluble, Partially *N*-Acetylated Chitosan with Lysozyme", *Carbohyd. Polym.*, **1994**, *23*, 253-260.
35. Tanioka, S.; Matsui, Y.; Irie, T.; Tanigawa, T.; Tanaka, Y.; shibata, H.; Sawa, Y., and Kono, Y., "Oxidative Depolymerization of Chitosan by Hydroxyl Radical", *Biosci. Biotech. Biochem.*, **1996**, *60*, 2001-2004.
36. Skjak-Braek, G.; Anthonsen, T., and Sanford, P., *Chitin and Chitosan*, London: Elsevier, **1989**, pp. 373-382.
37. Amano, K. and Ito, E., "The action of lysozyme on partially deacetylated chitin", *J. Biochem.*, **1987**, *85*, 97-104.
38. Zhu, B.C.R. and Laine, R.A., "Depolymerization of chitin with chitinase", *Chitin Handbook*, **1997**, 147-151.
39. Terayama, H.; Tarkahashi, S. and Kuzuhara, H., "", *J. Carbohydra. Chem.*, **1993**, *12*, 81-93.
40. Matsuoka, K.; Matsuzawa, Y.; Kusano, K.; Terunuma, D. and Kuzahara, H., "An improved preparation of *N,N'*-diacetylchitobiose by continuous enzymatic degradation of colloidal chitin using dialysis tubing as a convenient separator", *Biomacromolecules.*, **2000**, *1*, 798-800.
41. Takiguchi, Y., and Shimahara, K., "Isolation and Identification of A Thermophilic Bacterium Producing *N,N'*-Diacetylchitobiose from Chitin", *Agric. Biol. Chem.*, **1989**, *53*, 1537.
42. Takayanagi, T.; Ajisaka, K.; Takiguchi, Y., and Shimahara, K., "Isolation and Characterization of Thermostable Chitinases from *Bacillus licheniformis* X-7u", *Biochim. Biophy. Acta,* **1991**, *1078*, 404-410.

43. Mitsutomi, M.; Kidoh, H.; Tomita, H., and Watanabe, T., "The action of *Bacillus-circulans WL-12* Chitinases on Partially *N*-Acetylated Chitosan", *Biosci. Biotech. Biochem.*, **1995**, 59, 529-531.

44. Ohtakara, A.; Matsunaga, H., and Mitsutomi, M., "Action Pattern of *Streptomyces griseus* Chitinase on Partially *N*-Acetylated Chitosan", *Agric. Biol. Chem.*, **1990**, *54*, 3191-3199.

45. Mitsutomi, M.; Ohtakara, A.; Fukamizo, T., and Goto, S., "Action Pattern of *Aeromonas hydrophila* Chitinase on Partially *N*-Acetylated Chitosan", *Agric. Biol. Chem.*, **1990**, 54, 871-877.

46. Aiba, S., "Preparation of *N*-acetylchitooligosaccharides by Hydrolysis of Chitosan with Chitinase Followed by *N*-Acetylation", *Carbohyd. Res.*, **1994**, *265*, 323-328.

47. Fenton, M., and Eveleigh, D. E., "Purification and Mode of Action of A Chitosanase from *Penicillium islandium*", *J. Gen. Microbiol.*, **1981**, *126*, 151-165.

48. Izume, M.; Nagae, S.; Kawagishi, H., and Ohtakara, A., "Preparation of *N*-Acetylchitooligosaccharides from Enzymatic Hydrolyzates of Chitosan", *Biosci. Biotech. Biochem.*, **1992**, *56*, 1327-1328.

49. Kobayashi. S.; Kiyosada, T., and Shoda, S. I., "A Novel Method for Synthesis of Chitobiose *via* Enzymatic Glycosylation Using A Sugar Oxazoline as Glycosyl Donor", *Tetrahedron Letters*, **1997**, *38*, 2111-2112.

50. Domard, A.; Jeuniaux, C; Muzzarelli, R. A. A., and Roberts, G. A. F., *Advances in Chitin Science*" Lyon: Andre Publ., **1996**, pp.192-197.

51. Muzzarelli, R. A. A.; Xia, W.; Tomasetti, M., and Ilari, P., "Depolymerization of Chitosan and Substituted Chitosans with The Aids of A Wheat-Germ Lipase Preparation", *Enzyme Microb. Technol.*, **1995**, *17*, 541.

52. Naranong, N., "Production and characterization of chitinolytic enzyme from microorganism", *Ph.D. Thesis Biotechnology Department Kasetsart University*, **1997**.

53. Shashiwa, H.; Fujishima, S.; Yamana, N.; Kawasaki, N.; Nakayama, A.; Muraki, E., and Aiba, S., "Production of *N*-Acetyl-D-Glucosamine from β-Chitin by Enzymatic Hydrolysis", *Chem. Lett.*, **2001**, 308-309.

54. Sukwattanasinitt, M.; Zhu, H.; Shashiwa, H., and Aiba, S., "Utilization of Commercial Non-Chitinase Enzymes from Fungi for Preparation of 2-Acetamido-2-Deoxy-D-Glucose from β-Chitin", *Carbohyd. Res.*, **2002**, *337*, 133-137.

55. Sashiwa, H.; Fujishima, S.; Yamano, N.; Kawasaki, N.; Nakayama, A.; Muraki, E.; Hiraga, K.; Oda, K., and Aiba, S., "Production of *N*-Acetyl-D-Glucosamine from α-Chitin by Crude Enzymes from *Aeromonas hydrophila* H-2330", *Carbohyd. Res.*, **2002**, *337*, 761-763.

56. Pichyangkura, R.; Kudan, S.; Kuttiyawong, K.; Sukwattanasinitt, M., and Aiba, S., "Quantitative Production of 2-Acetamido-2-Deoxy-D-Glucose from Crystalline Chitin by Bacterial Chitinase", *Carbohyd. Res.*, **2002**, *337*, 557-559.

57. Ilkovan, P.; Hein, S.; Ng, C.; Trung, T.S. and Stevens, W.F., "Production of N-acetyl chitobiose from various chitin substrates using commercial enzymes", *Carbohydrate Polymers*, **2005**, *63(2)*, 245-250.

58. Klaikherd, A.; Jayanta. M.L.S.; Boonjawat, J.; Aiba, S. and Sukwattanasinitt, M., "Depolymerization of β-chitin to mono- and disaccharides by the serum fraction from the *para* rubber tree, *Hevea brasiliensis*", *Carbohydrate Research*, **2004**, *339*, 2799-2804.

59. Auynirundornkul, K., "Production of aminosugar from squid pen chitin by fungal biocatalyst", *Master Thesis of Science in Petrochemistry and polymer Program Petrochemistry and polymer science Faculty of Science Chulalongkorn University*, **2003**.

60. Prakobkij, W., "Preparation of *N,N'*-diacetylchitobiose from chitin by enzymatic hydrolysis", *Master Thesis of Science in Petrochemistry and polymer Program Petrochemistry and polymer science Faculty of Science Chulalongkorn University*, **2003**.

61. Aiba, S. and Muraki, E., "Advances in chitin science" (A. Domard, C. Jeuniaux, R.A.A. Muzzarelli and G.A.F. Roberts, eds), **1996**, Andre Publ., Lyon., 192-197.

62. Jeon, Y. J., and Kim, S. K., "Production of Chitooligosaccharides Using An Ultrafiltration Membrane Reactor and Their Antibacterial Activity", *Carbohyd. Polym.*, **2000**, *41*, 133-141.

63. Matsuoka, K.; Matsuzawa, Y.; Kusano, K.; Terunuma D., and Kuzuhara, H., "An Improved Preparation of *N,N'*-Diacetylchitobiose by Continuous Enzymatic Degradation of Colloidal Chitin Using Dialysis Tubing as A Convenient Separator", *Biomacromolecules*, **2000**, *1*, 798-800.

64. Haynes, C.A.; Aloise, P. and Creagh, A.L., "Process for producing *N*-acetyl-D-glucosamine", **1999**, United States Patent: 5,998,173.

65. Heftmann, E., "Chapter 4 Adsorption", *Chromatography second edition*, Litton Educational Publishing, Inc., New York, **1967**, 43-44.

66. Yoon, J.H., "Enzymatic synthesis of chitooligosaccharides in organic cosolvents", *Enzyme and Microbial Technology*, **2005**, *37*, 663-668.

67. Nishimura, S.; Kuzuhara, H.; Takiguchi, Y., and Shimahara, K, "Peracetylated Chitobiose: Preparation by Specific Degradations of Chitin, and Chemical Manipulations", *Carbohydr. Res.*, **1989**, *194*, pp. 223-231.

68. Kuttiyawong, K., "Cloning and nucleotide sequencing of chitinase gene from Burkholderia cepacia TU09", *Master Thesis of Science, Biochemistry, Faculty of Science Chulalongkorn University*, **2001**.

69. Imoto, T., and Yagishita, K., "A Simple Activity Measurement of Lysozyme", *Agr. Biol. Chem.* **1971**, *35*, 1154-1156.

APPENDICES

APPENDIX A

1. Prepartion calibration curve of N-acetyl-D-glucosamine for chitinolytic enzyme assay by colorimetric method.

Calibration curve for GlcNAc was made by determining the absorbance value at 420 nm of standard GlcNAc according to the method of Schales.

Table A1 The amount of standard solution of GlcNAc and ΔAbsorbance

standard No.	amount of GlcNAc (μmole)	ΔAbsorbance
1	0.6021	0.725
2	0.5018	0.599
3	0.4837	0.578
4	0.3870	0.468
5	0.2902	0.354
6	0.1935	0.204
7	0.0967	0.091

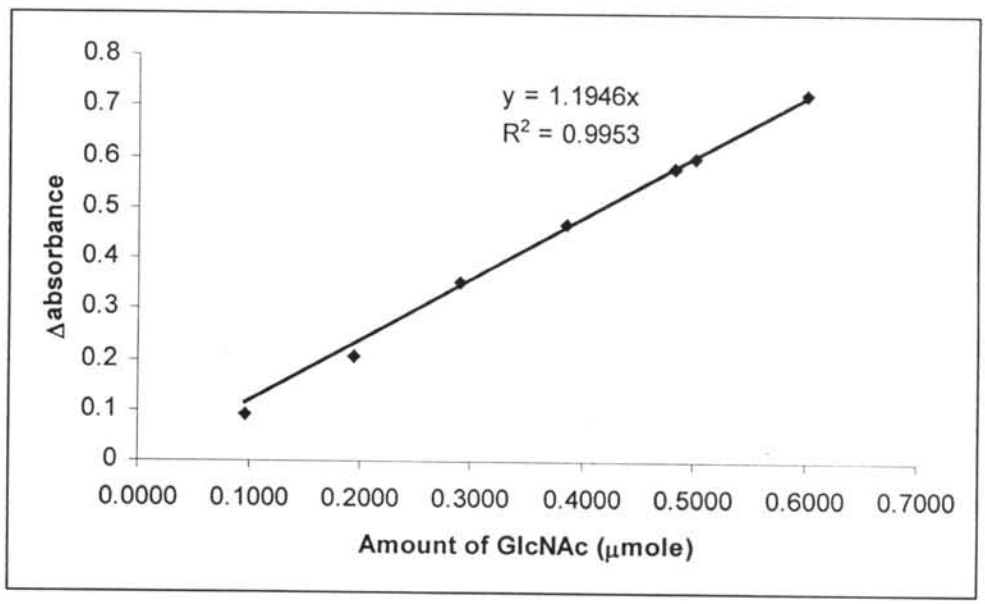


Figure A1 Correlation between amount of standard of *N*-acetyl-D-glucosamine and optical density (absorbance) at 420 nm

2. Preparation the calibration curve of *N*-acetyl-D-glucosamine for HPLC analysis

Calibration curve of GlcNAc was made by varying the concentration and measuring the peak area by HPLC.

Table A2 The concentration of standard solution of GlcNAc and peak area.

Standard No.	Conc. GlcNAc (mM)	Peak Area (mV*Sec)
1	0.12	51.953
2	0.21	92.341
3	0.59	251.211
4	1.07	453.525
5	2.36	1007.929
6	3.20	1383.540

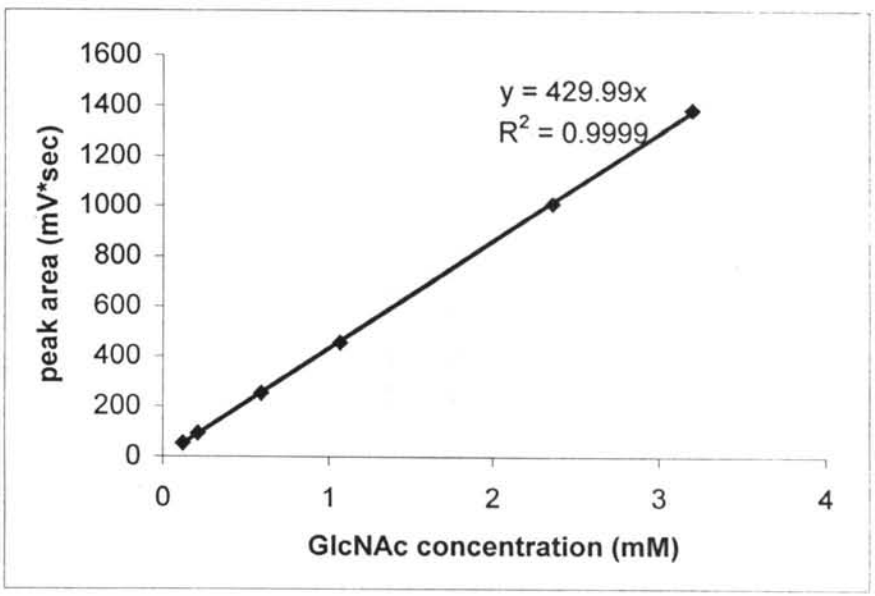


Figure A2 Correlation between concentration of standard *N*-acetyl-D-glucosamine and peak area by HPLC.

3. Preparation the calibration curve of *N,N'*-diacetylchitobiose for HPLC analysis

Calibration curve of $(GlcNAc)_2$ was made by varying the concentration and measuring the peak area by HPLC.

Table A3 The concentration of standard solution of $(GlcNAc)_2$ and peak area.

Standard No.	Conc. GlcNAc (mM)	Peak Area (mV*Sec)
1	0.06	37.613
2	0.11	100.833
3	0.30	192.349
4	0.55	391.312
5	1.20	776.494
6	1.65	1191.126

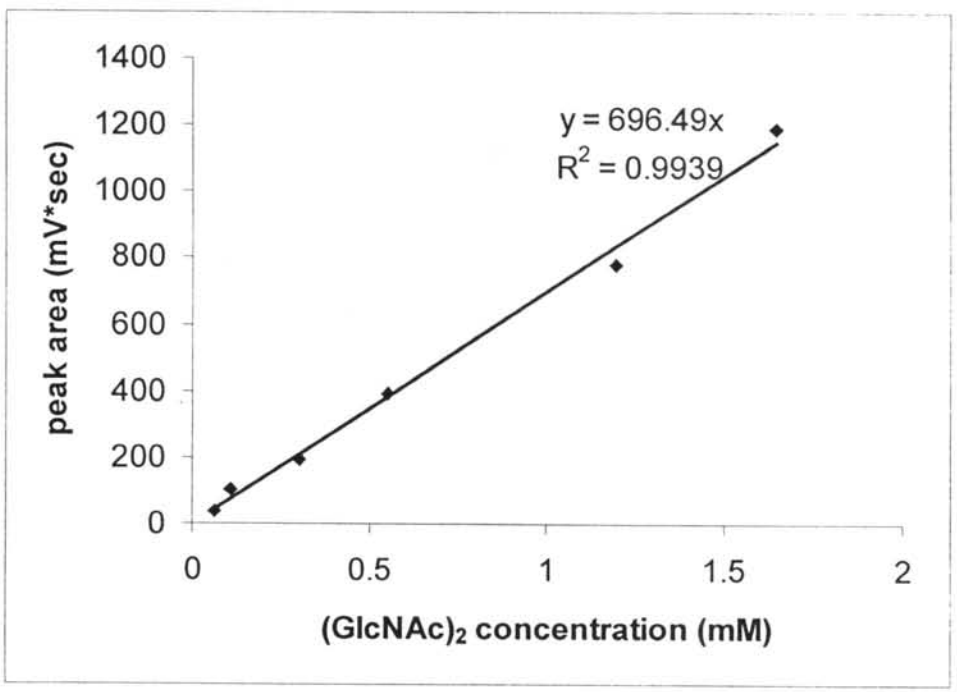


Figure A3 Correlation between concentration of standard *N,N'*-diacetylchitobiose and peak area by HPLC.

APPENDIX B

1. Precipitation of standard GlcNAc by using absolute ethanol, acetone and acetonitrile

Table B3 Pecipitation of standard GlcNAc by absolute ethanol at various ratios

H_2O:EtOH	Precipitate weight (mg)	%precipitate
1:1	77.1	
	73.7	
	75.4	**54**
1:2	96.4	
	96.1	
	96.3	**69**
1:5	115.5	
	114.9	
	115.2	**82**
1:10	107.6	
	101.6	
	104.6	**75**
1:15	111.8	
	112.0	
	111.9	**80**

Various volume of EtOH was added into saturated standard GlcNAc (140 mg) solution (0.5 mL) in water. The average value (bold font) was plotted in the graph **Table 3.1**.

Table B4 Pecipitation of standard GlcNAc by acetone at various ratios

H_2O:acetone	precipitate weight (mg)	%precipitate
1:1	9.3	9.6
1:2	8.1	8.3
1:4	53.0	54
1:8	57.8	59
1:15	67.6	69

Various volume of dried acetone was added into saturated standard GlcNAc (97 mg) solution (0.5 mL) in water.

Table B5 Pecipitation of standard GlcNAc by acetonitrile at various ratios

H_2O:acetonitrile	precipitate weight (mg)	%precipitate
1:1	0	0
1:2	0	0
1:4	12.8	13
1:8	18.5	19
1:15	19.4	20

Various volume of dried acetonitrile was added into saturated standard GlcNAc (97 mg) solution (0.5 mL) in water.

2. Purification of GlcNAc and $(GlcNAc)_2$ by precipitation technique

Table B6 Amount of GlcNAc in precipitate and supernatant obtained by absolute EtOH precipitation.

H_2O:EtOH	precipitate		supernatant	
	weight (mg)	%purity	weight (mg)	%purity
1:1	19.9	55	105.9	77
	19.5	48	109.0	69
	19.7	**52**	**107.5**	**73**
1:2	17.0	66	113.6	72
	18.6	66	113.4	73
	17.8	**66**	**113.5**	**73**
1:5	13.1	49	115.2	73
	14.9	59	115.2	74
	14.0	**54**	**115.2**	**74**
1:10	18.8	64	109.8	79
	18.9	67	109.8	78
	18.9	**66**	**109.8**	**79**
1:15	15.3	41	113.6	78
	15.2	49	112.5	80
	15.3	**45**	**113.1**	**79**

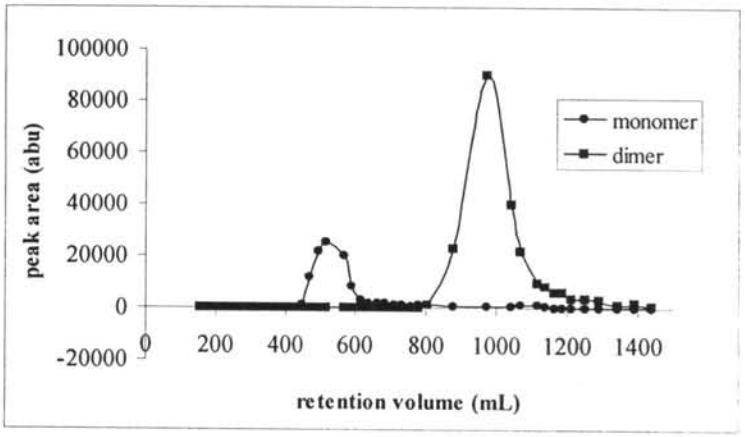
Various volume of EtOH was added into saturated standard GlcNAc (158 mg) solution (0.5 mL) in water. The average value (bold font) was plotted in the graph **Table 3.2**.

Table B7 Amount of $(GlcNAc)_2$ in precipitate and supernatant obtained by absolute EtOH precipitation.

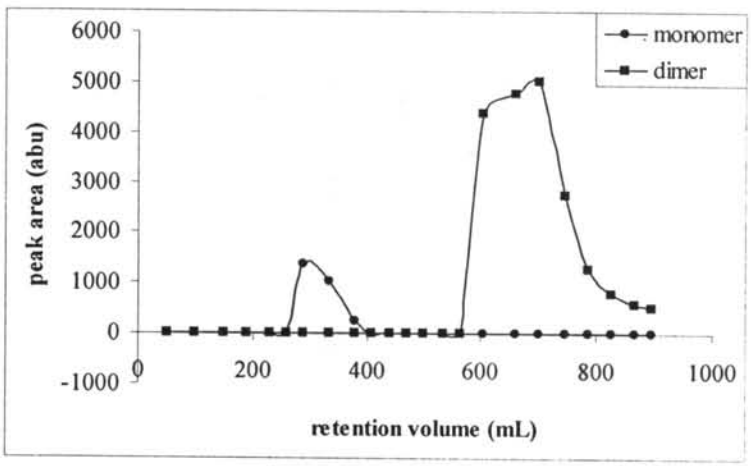
H_2O:EtOH	precipitate		supernatant	
	weight (mg)	**%purity**	**weight (mg)**	**%purity**
1:1	14.4	44	92.8	47
	15.5	42	94.3	47
	15.0	**43**	**93.6**	**47**
1:2	16.2	38	94.8	43
	16.0	35	93.4	48
	16.1	**37**	**94.1**	**46**
1:5	14.0	30	86.1	53
	14.0	28	89.5	51
	14.0	**29**	**87.8**	**52**
1:10	14.5	23	94.6	49
	14.7	20	92.8	55
	14.6	**22**	**93.7**	**52**
1:15	18.0	27	86.2	57
	18.3	28	86.0	60
	18.2	**28**	**86.1**	**59**

Various volume of EtOH was added into saturated standard GlcNAc (106 mg) solution (0.5 mL) in water. The average value (bold font) was plotted in the graph **Figure3.2**.

3. Purification of $(GlcNAc)_2$ by activated charcoal column: determination of loading capacity of charcoal.



(a)



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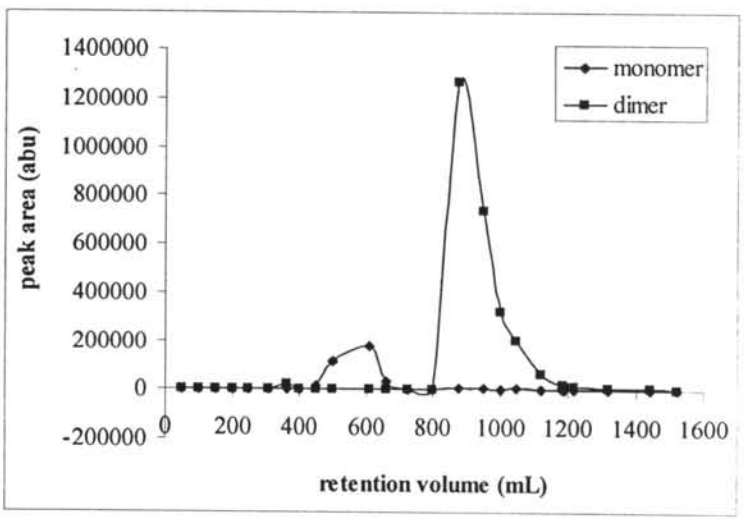

(c)

Figure A1 chromatogram of GlcNAc and $(GlcNAc)_2$ from activated charcoal column with loading sugars of 0.44 g (a), 0.89 g (b) and 1.86 g (c)

VITAE

Miss Thitima Maneekul was born on June 19th, 1981 in Nakhornpathom, Thailand. She received a Bachelor Degree of Science, majoring in Chemistry from Chulalongkorn University, in 2002. Since 2003, she has been a graduate student studying Organic Chemistry as her major course at Chulalongkorn University. During her studies towards the Master's Degree, she was awarded a teaching assistant scholarship by the Faculty of Science during 2003-2004.

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