

การเตรียมเอ็น,เอ็น-ไดแอซิทิลโคโทไบโอสจากโคทินโดยการย่อยด้วยเอนไซม์

นางสาว วาสนี ประกอบกิจ

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

สาขาวิชาปิโตรเคมีและวิทยาศาสตร์พอลิเมอร์

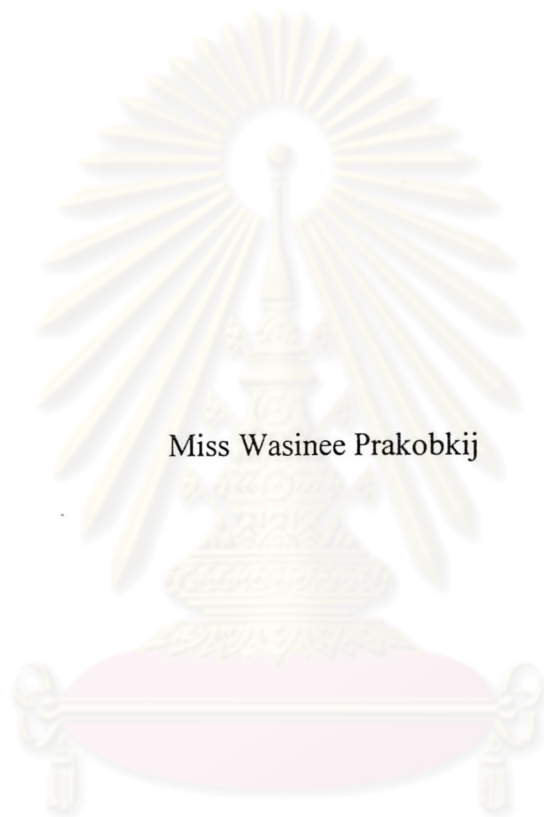
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ปีการศึกษา 2546

ISBN 974-17-3728-9

ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

PREPARATION OF *N,N'*-DIACETYLCHITOBIOSE FROM CHITIN BY
ENZYMATIC HYDROLYSIS




Miss Wasinee Prakobkij

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย


A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Petrochemistry and Polymer Science
Faculty of Science
Chulalongkorn University
Academic Year 2003
ISBN 974-17-3728-9

Thesis Title Preparation of *N,N'*-diacetylchitobiose from chitin by enzymatic hydrolysis
By Miss Wasinee Prakobkij
Field of Study Petrochemistry and Polymer Science
Thesis Advisor Assistant Professor Mongkol Sukwattanasinitt, Ph.D.
Thesis Co-Advisor Rath Pichyangura, Ph.D.


Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

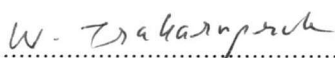

..... Dean of Faculty of Science
(Associate Professor Wanchai Phothiphichitr, Ph.D.)

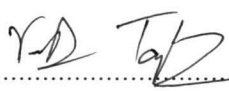
THESIS COMMITTEE


..... Chairman
(Professor Pattarapan Prasassarakich, Ph.D.)


..... Thesis Advisor
(Assistant Professor Mongkol Sukwattanasinitt, Ph.D.)


..... Thesis Co-Advisor
(Rath Pichyangura, Ph.D.)


..... Member
(Associate Professor Wimonrat Trakarnpruk, Ph.D.)


..... Member
(Varawut Tangpasuthadol, Ph.D.)

วาทินี ประกอบกิจ: การเตรียมเอ็น,เอ็น-ไดอะซิทิลโคโทไบโอสจากไคตินโดยการย่อยด้วยเอนไซม์ (PREPARATION OF *N,N'*-DIACETYLCHITOBIOSE FROM CHITIN BY ENZYMATC HYDROLYSIS) อ.ที่ปรึกษา: ผศ. ดร. มงคล สุขวัฒนาสินิทธิ; อ.ที่ปรึกษา
ร่วม: อ. ดร. รัฐ พิษญากร; 90 หน้า. ISBN 974-17-3728-9

ได้ศึกษาเอนไซม์ 2 ชนิดได้แก่ cellulase *Ac* จาก *Acremonium cellulolyticus* และ Chi 60 จาก *Serratia* sp. เพื่อใช้ในการเตรียมน้ำตาลเอ็น,เอ็น-ไดอะซิทิลโคโทไบโอสจากไคติน ในงานวิจัยนี้ได้ทำการศึกษาสภาวะที่เหมาะสมต่อการย่อยของเอนไซม์ทั้ง 2 ชนิด การศึกษาสภาวะที่เหมาะสมต่อการย่อยของเอนไซม์ cellulase *Ac* ได้ใช้ซับสเตรท 2 ชนิดคือ ไคตินผงและไคตินเส้นใย ความแตกต่างของสภาวะที่เหมาะสมสำหรับซับสเตรท 2 ชนิดนี้คือ ความเข้มข้นของไคตินและความเข้มข้นของเอนไซม์ สำหรับเอนไซม์ cellulase *Ac* อัตราส่วนโมลผลิตภัณฑ์ [(GlcNAc)₂/GlcNAc] สามารถปรับให้เพิ่มขึ้นจาก 1 เป็นมากกว่า 3 ได้โดยใช้เทคนิคการดูดซับเอนไซม์ ซึ่งแสดงว่าเอนไซม์ cellulase *Ac* มีส่วนประกอบเอนไซม์ทั้งที่เป็น chitinase และ chitobiase ในการศึกษาสภาวะที่เหมาะสมต่อการย่อยของเอนไซม์ Chi 60 ได้ใช้เพียงไคตินเส้นใยเป็นซับสเตรทเท่านั้น การใช้ความเข้มข้นของเอนไซม์ Chi 60 ต่ำๆ (น้อยกว่า 30 มิลลิยูนิตต่อ 1 มิลลิลิตร) ให้ผลิตภัณฑ์เป็นน้ำตาลเอ็น,เอ็น-ไดอะซิทิลโคโทไบโอสเพียงชนิดเดียวเท่านั้น แต่ที่ความเข้มข้นของ Chi 60 สูงๆ มีผลิตภัณฑ์ทั้ง (GlcNAc)₂ และ GlcNAc เกิดขึ้น เทคนิคการดูดซับเอนไซม์ไม่สามารถใช้ในการเพิ่มอัตราส่วนโมลผลิตภัณฑ์ได้สำหรับเอนไซม์นี้แสดงว่าเอนไซม์ Chi 60 น่าจะประกอบด้วยเอนไซม์เพียงชนิดเดียวที่มีความสามารถในการย่อยทั้งภายในและปลายโซ่ไคตินได้

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

สาขาวิชา ปีโตรเคมีและวิทยาศาสตร์พอลิเมอร์.....ลายมือชื่อนิสิต..... วาณิณี ประกอบกิจ.....
ปีการศึกษา..... 2546.....ลายมือชื่ออาจารย์ที่ปรึกษา.....
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

4372409323: MAJOR PETROCHEMISTRY AND POLYMER SCIENCE

KEY WORDS : β -CHITIN / *N,N'*-DIACETYLCHITOBIOSE / ENZYMATIC HYDROLYSIS

WASINEE PRAKOBKIJ: PREPARATION OF *N,N'*-DIACETYLCHITOBIOSE FROM CHITIN BY ENZYMATIC HYDROLYSIS. THESIS ADVISOR: ASSIST. PROF. MONGKOL SUKWATTANASINITT, Ph.D.; THESIS CO-ADVISOR: RATH PICHYANGKURA, Ph.D., 90 pp. ISBN 974-17-3728-9

Two types of enzymes, cellulase *Ac* from *Acremonium cellulolyticus* and Chi 60 from *Serratia* sp, were studied for a preparation of *N,N'*-Diacetylchitobiose from chitin. The optimum conditions for both enzymes to hydrolyzed chitin were investigated in this work. In study for the optimum condition of cellulase *Ac*, powder chitin and fibrous chitin were used as the substrates. The difference optimum conditions of both substrates were chitin concentration and enzyme concentration. The (GlcNAc)₂/GlcNAc mole ratio can be improved from 1 to over 3 by using the enzyme affinity technique, indicating that cellulase *Ac* contained both chitinase and β -N-acetylhexosaminidase. For Chi 60, only fibrous chitin was used as a substrate. At lower concentration of Chi 60 (< 30 mU/mL), the chitinolytic product was only (GlcNAc)₂ but at higher concentration of the enzyme, both (GlcNAc)₂ and GlcNAc were observed. The enzyme affinity technique cannot be used to improve the (GlcNAc)₂/GlcNAc mole ratio for Chi 60 indicating that this enzyme consists of only one enzyme which had both endo- and exo-chitinolytic activities.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Field of study.. Petrochemistry and Polymer Science...Student's signature... *P. Wasinee*

Academic year.....2003.....Advisor's signature... *Asst. Prof. Mongkol Sukwattanasinitt*

Co-advisor's signature..... *Rath Pichyangkura*

ACKNOWLEDGEMENT

I would like to express my gratitude and appreciation to my advisors, Asst. Prof. Dr. Mongkol Sukwattanasinitt and Dr. Rath Pichyangkura, for their helpful suggestions, generous guidance, and encouragement throughout this research. Gratefully thanks to Professor Dr. Pattarapan Prasassarakich, Associate Professor Dr. Wimonrat Trakarnpruk and Dr. Varawut Tangpasuthadol, for their valuable suggestions and advice as thesis chairman and committee.

I would like to gratefully thank Miss Kitsana Siralerdmukun from Metallurgy and Materials Science Research for permission to use an ultracentrifugal mill. I would like to extend my thank to M.L. Siripastr Jayanta for invaluable advices on HPLC techniques. I would also like to thank Miss Kamolthip Kuttiyawong and Miss Santhana Nakatong at Department of Biochemistry, Faculty of Science, Chulalongkorn University for their helps in biochemistry laboratory. Many thanks to the MS-group's members: Panithan, Siriporn, Akamol, Krissana, Anupat, Arisa, and Chantana for their helps in everything. I would also like to thank the Graduate School, Chulalongkorn University for research financial support.

This work is part of department of chemistry OECF-JBIT project and financially by National Metal and Materials Technology center (MTEC), project code MT-B-46-POL-09-206-6.

Finally, I also would like to express my gratitude to my family for their love, support and encouragement. Especially, Pradhammarachanuwat who financially support me in this higher education study. Without their love, support and kindness this thesis could not have been succeeded.

CONTENTS

	Page
Abstract in Thai.....	iv
Abstract in English.....	v
Acknowledgement	vi
List of Figures.....	xi
List of Tables	xiii
List of Abbreviation.....	xiv

CHAPTER I: INTRODUCTION

1.1 Chitin chemistry	1
1.2 The structure of chitin.....	2
1.3 Manufacture of chitin.....	4
1.4 Applications of chitin.....	4
1.5 The use of monomers and oligomers of chitin and chitosan	5
1.6 Hydrolysis of chitin	9
1.6.1 Chemical hydrolysis.....	9
1.6.2 Enzymatic hydrolysis.....	11
1.7 Chitinase	13
1.8 Substrate binding mechanism of chitinolytic enzyme	14
1.9 <i>Serratia</i> sp.....	15
1.10 <i>Acremonium</i> sp.....	17
1.11 Aims of thesis	18

CHAPTER II: EXPERIMENTS

Instruments and Apparatus	19
Chemicals.....	20
General Procedure.....	21
2.1 Preparation and characterization of substrates and enzymes.....	21
2.1.1 Squid pen chitin 100 μ m.....	21
2.1.2 Colloidal chitin	21

CONTENTS (Continued)

	Page
2.1.3 Protein assaying of enzyme	21
2.1.3.1 Preparation of calibration curve.....	21
2.1.3.2 Measurement of the protein amount in the Chi 60	22
2.1.4 Chitinase activity assaying.....	22
2.1.4.1 Preparation of the calibration curve.....	22
2.1.4.2 Activity assaying.....	23
2.2 Study of hydrolytic products by HPLC	25
2.2.1 Preparation of calibration curve for <i>N</i> -acetyl-D-glucosamine.....	25
2.2.2 Preparation of calibration curve for <i>N,N'</i> -diacetylchitobiose	26
2.2.3 Analysis of the hydrolytic products	26
2.3 Study for the optimum condition for the enzymatic hydrolysis with cellulase <i>Acremonium cellulolyticus</i>	27
2.3.1 Concentration of chitin	27
2.3.2 Concentration of cellulase <i>Ac</i>	28
2.3.3 pH of the reaction solution.....	28
2.3.4 Concentration of buffer	29
2.3.5 Temperature	29
2.3.6 Enzyme affinity technique	29
2.4 Study for the optimum condition for the enzymatic hydrolysis with chitinase <i>Serratia sp.</i> Cloned (Chi 60).....	30
2.4.1 Concentration of chitin	30
2.4.2 Concentration of chitinase	30
2.4.3 pH of the reaction solution.....	31
2.4.4 Concentration of buffer	31
2.4.5 Temperature	32
2.4.6 Enzyme affinity technique	32
2.4.7 Preparative scale preparation of <i>N,N'</i> -diacetylchitobiose	33
2.4.8 Hydrolysis of (GlcNAc) ₂	33

CONTENTS (Continued)

	Page
CHAPTER III: RESULTS AND DISCUSSIONS	
3.1 Substrates and Enzymes.....	34
3.1.1 Fibrous squid pen chitin.....	34
3.1.2 Colloidal chitin	35
3.1.3 Protein assaying of enzyme	35
3.1.4 Chitinase activity assaying.....	35
3.2 Study of hydrolytic products by HPLC	35
3.3 Study for the optimum condition in the enzymatic hydrolysis with cellulase <i>Acremonium cellulolyticus</i>	36
3.3.1 Concentration of chitin	36
3.3.2 Concentration of cellulase <i>Ac</i>	38
3.3.3 pH of the reaction solution.....	41
3.3.4 Concentration of buffer.....	43
3.3.5 Temperature	44
3.3.6 Enzyme affinity technique	46
3.4 Study for the optimum condition in the enzymatic hydrolysis with chitinase <i>Serratia sp.</i> Cloned (Chi 60).....	47
3.4.1 Concentration of chitin	47
3.4.2 Concentration of chitinase	48
3.4.3 pH of the reaction solution.....	50
3.4.4 Concentration of buffer.....	51
3.4.5 Temperature	52
3.4.6 The enzyme affinity technique.....	53
3.4.7 Gram scale preparation of <i>N,N'</i> -diacetylchitobiose	54
3.4.8 Hydrolysis of (GlcNAc) ₂	55
CHAPTER IV: CONCLUSIONS	
REFERENCES	58
APPENDICES	65

APPENDIX A.....	66
APPENDIX B.....	72
VITAE.....	90



ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

LIST OF FIGURES

Figure	Page
1.1 Chemical structures of (a) chitin and (b) cellulose	1
1.2 Diagrammatic illustration of antiparallel arrangement and X-ray crystal structure displaying hydrogen bond linkage between to C=O and NH groups of α -chitin.....	3
1.3 Diagrammatic illustration of antiparallel arrangement and X-ray crystal structure displaying hydrogen bond linkage between to C=O and NH groups of β -chitin.....	3
1.4 Simplified flowsheet for preparation of chitin and chitosan, their monomers and oligomers from invertebrate marine's waste.....	7
1.5 Reaction for acid hydrolysis of chitin.....	10
1.6 Pathway for conversion of chitin and chitosan into their oligomers by enzymatic means.....	11
1.7 Preparation the (GlcNAc) ₂ by enzymatic transglycosylation	12
1.8 Structure of chitinase Chi 60, (a) Structure of <i>Serratia marcescens</i> ChiA displayed by Rasmol 2.6 and (b) Theoretical model of <i>Serratia sp.</i> Chi 60 accomplished by Swiss-Model Protein Modeling (SWISS-MODEL version 36.0002)	16
3.1 The squid pen chitin, (a) flake chitin, (b) fibrous β -chitin 100x50 μ m (1x)....	34
3.2 The effect of chitin concentration on the chitinolysis of powder chitin with cellulase <i>Ac</i>	37
3.3 The effect of chitin concentration on the chitinolysis of fibrous chitin with cellulase <i>Ac</i>	38
3.4 The effect of enzyme concentration on the chitinolysis of powder chitin with cellulase <i>Ac</i>	39
3.5 The effect of enzyme concentration on the chitinolysis of fibrous chitin with cellulase <i>Ac</i>	41
3.6 The effect of pH on (a) yields of products and (b) product mole ratio.....	42
3.7 The effect of concentration of buffer on (a) yields of products and (b) product mole ratio	44

LIST OF FIGURES (Continued)

Figure	Page
3.8 The effect of temperature on the chitinolysis of fibrous chitin with cellulase <i>Ac</i>	45
3.9 The effect of the chilling time on the chitinolysis of powder chitin with Cellulase <i>Ac</i> using enzyme adsorption technique	46
3.10 The effect of chitin concentration on the chitinolysis of fibrous chitin with Chi 60.....	48
3.11 The effect of enzyme concentration on the chitinolysis of fibrous chitin with Chi 60.....	49
3.12 The effect of pH on the chitinolysis of fibrous chitin with Chi 60.....	50
3.13 The effect of concentration of buffer on the chitinolysis of fibrous chitin with Chi 60.....	51
3.14 The effect of temperature on the chitinolysis of fibrous chitin with Chi 60....	53
3.15 The effect of enzyme adsorption on the chitinolysis of fibrous chitin with Chi 60.....	54

LIST OF TABLES

Tables	Pages
1.1 Applications of chitin, chitosan, their monomers and oligomers	8
2.1 Preparation of the protein standard solutions	22
2.2 Concentration of GlcNAc and standard solutions for enzyme assaying	23
2.3 Preparation of GlcNAc standard solutions	25
2.4 Preparation of (GlcNAc) ₂ standard solutions	26
2.5 The amounts of reagents for hydrolysis of chitin with enzyme chitinase from cellulase <i>Ac</i>	27
2.6 The amounts of reagents used in the study for the optimum concentration of chitinase from cellulase <i>Ac</i>	28
2.7 The amounts of reagents used in the reaction in the study for suitable concentration buffers	29
2.8 The amounts of reagents for hydrolysis of chitin with enzyme chitinase from Chi 60	30
2.9 The amounts of enzyme and water needed in each reaction	31
2.10 The amounts of reagents used in the reaction in the study for optimum buffer concentration	32
2.11 The amounts of reagents used in the reaction in the study for hydrolysis of (GlcNAc) ₂	33
3.1 The effect of enzyme adsorption on the chitinolysis of fibrous chitin with cellulase <i>Ac</i> in comparison with non adsorption	47
3.2 The hydrolysis of (GlcNAc) ₂ with cellulase <i>Ac</i>	55
3.3 The hydrolysis of (GlcNAc) ₂ with Chi 60	55

LIST OF ABBREVIATIONS

°C	degree Celcius	nm	nanometre
DI-water	deionized water	Sec	second
g	gram (s)	U	unit
GlcNAc	<i>N</i> -acetyl-D-glucosamine	α	alpha
(GlcNAc) ₂	<i>N,N'</i> -diacetylchitobiose	β	beta
HPLC	high performance liquid chromatography	μ g	microgram
hr	hour	μ L	microlitre
min	minute	μ m	micrometre
mg	milligram	μ M	micromolar
mL	millilitre	%	percent
mM	millimolar		

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย