การปรับปรุงแอกทิวิตีในการสังเคราะห์ออลิโกแซ็กคาไรด์ของบีตากาแลกโทซิเดสในตัวทำละลายอินทรีย์โดยใช้
ไซโคลเดกซ์ทริน

นางสาววิรยา ศรีสิมารัตน์

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ปีการศึกษา 2549

ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

IMPROVEMENT OF OLIGOSACCHARIDE SYNTHETIC ACTIVITY
OF β-GALACTOSIDASE IN ORGANIC SOLVENTS
BY USING CYCLODEXTRINS

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สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

A Thesis Submitted in Partial Fulfillment of the Requirements
For the Degree of Master of Science Program in Biochemistry
Department of Biochemistry
Faculty of Science
Chulalongkorn University
Academic Year 2006
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Thesis title	IMPROVEMENT OF OLIGOSACCHARIDE SYNTHETIC ACTIVITY OF β-GALACTOSIDASE IN ORGANIC SOLVENTS BY USING CYCLODEXTRINS
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วิรยา ศรีสิมารัตน์ : การปรับปรุงแอกทิวิตีในการสังเคราะห์ออลิโกแซ็กคาไรด์ของบีตากาแลกโทซิเดสในตัวทำละลายอินทรีย์โดยใช้ไซโคลเดกซ์ทริน (IMPROVEMENT OF OLIGOSACCHARIDE SYNTHETIC ACTIVITY OF β-GALACTOSIDASE IN ORGANIC SOLVENTS BY USING CYCLODEXTRINS) อ. ที่ปรึกษา : รศ.ดร.เปี่ยมสุข พงษ์สวัสดิ์; 94 หน้า.

ในการสังเคราะห์ออลิโกแซ็กคาไรด์จากแลกโทสโดยใช้บีตากาแลกโทซิเดสจาก *Aspergillus oryzae* และทำปฏิกิริยาในตัวทำละลายอินทรีย์ผสมน้ำ พบว่า ตรวจพบผลิตภัณฑ์ออลิโกแซ็กคาไรด์ (Oligo-I, Rf 0.19) โดยการวิเคราะห์ด้วย TLC เฉพาะในตัวทำละลายผสม triethyl phosphate (TEP) และ methoxyethyl acetate (MEA) ในขณะที่ไม่พบผลิตภัณฑ์ใน 1,4-butanediol, 1,5-pentanediol และ acetonitrile (ทุกกรณีใช้ตัวทำละลายอินทรีย์ผสมกับบัฟเฟอร์ซิเตรตในอัตราส่วนร้อยละ 50 โดยปริมาตร) เมื่อตรวจวัดปริมาณผลิตภัณฑ์ด้วยวิธี HPLC พบว่าผลิตภัณฑ์ที่สังเคราะห์ใน TEP ให้ผลผลิตมากกว่าการสังเคราะห์ในบัฟเฟอร์และ MEA เป็น 1.6 และ 6.8 เท่าตามลำดับ แต่อย่างไรก็ตาม ผลิตภัณฑ์ที่เกิดจากเอนไซม์ที่ไม่ได้ผ่านการทำแห้ง และทำการสังเคราะห์ในสารละลาย TEP ให้ผลผลิตเพียง 18.5% เท่านั้น เนื่องจากเอนไซม์มีความสามารถในการทำงานลดลงในภาวะที่มีตัวทำละลายอินทรีย์ จึงได้นำเอนไซม์ในบัฟเฟอร์ไปผ่านการทำแห้งร่วมกับไซโคลเดกซ์ทริน (CD) ซึ่งเป็นสารออลิโกแซ็กคาไรด์วงปิดขนาดใหญ่และเป็นสารจากภายนอกที่ใช้เติมในปฏิกิริยา พบว่า β-CD สามารถทำงานได้ดีที่สุดเมื่อเทียบกับอนุพันธ์ไฮดรอกซีโพรพิลและอนุพันธ์เมทิล โดย β-galactosidase/β-CD ในอัตราส่วน 1:5 โดยน้ำหนัก จะเพิ่มการสังเคราะห์ออลิโกแซ็กคาไรด์ใน 50% TEP ได้ 20.9% เมื่อเทียบกับการสังเคราะห์จากเอนไซม์ที่ทำแห้งในภาวะที่ไม่มี CD หรือเอนไซม์ที่ไม่ได้ผ่านการทำแห้ง ภาวะที่เหมาะสมที่สุดในการสังเคราะห์ออลิโกแซ็กคาไรด์ในสารละลาย 50% TEP โดยใช้เอนไซม์ทำแห้งร่วมกับ β-CD ในอัตราส่วน 1:5 คือ บ่มแลกโทส 0.3M กับเอนไซม์ 0.025 มิลลิกรัม ที่ pH 4.5, 40 °ซ เป็นเวลา 36-72 ชั่วโมง จะได้ผลิตภัณฑ์หลักคือ Oligo-I และ ผลิตภัณฑ์รองคือ Oligo-II ผลผลิตของ Oligo I จากการบ่มในภาวะที่เหมาะสมที่สุดได้ 31.6% ซึ่งเพิ่มขึ้นจากการใช้เอนไซม์ที่ทำแห้งแบบไม่ได้เติม CD และทำการสังเคราะห์ในภาวะที่ยังไม่ได้ปรับให้เหมาะสมถึง 63.6% ผลที่ได้แสดงว่า β-CD สามารถเพิ่มการสังเคราะห์ออลิโกแซ็กคาไรด์โดยใช้บีตากาแลกโทซิเดสในตัวทำละลายผสม TEP เมื่อนำผลิตภัณฑ์ที่สังเคราะห์ได้ไปวิเคราะห์ด้วย MS และ NMR พบว่า Oligo-I คือ 6'-galactosyl-lactose มีโครงสร้างเป็น *O*-β-D-galactopyranosyl-(1-6)-*O*-β-D-galactopyranosyl-(1-4)-D-glucopyranose ส่วน Oligo II พบว่าเป็นเตตระแซ็กคาไรด์ที่มีเพนตะแซ็กคาไรด์ปนอยู่

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ภาควิชา.....ชีวเคมี.....ลายมือชื่อนิสิต.....
สาขาวิชา.....ชีวเคมี.....ลายมือชื่ออาจารย์ที่ปรึกษา.....
ปีการศึกษา .....2549.....

4772477323 : MAJOR BIOCHEMISTRY
KEY WORD: β-GALACTOSIDASE / CYCLODEXTRIN / ORGANIC SOLVENT / OLIGOSACCHARIDE / ENZYMATIC SYNTHESIS

WIRAYA SRISIMARAT : IMPROVEMENT OF OLIGOSACCHARIDE SYNTHETIC ACTIVITY OF β-GALACTOSIDASE IN ORGANIC SOLVENTS BY USING CYCLODEXTRINS. THESIS ADVISOR : ASSOC.PROF. PIAMSOOK PONGSAWASDI, Ph.D. 94 pp.

Synthesis of oligosaccharides from lactose catalyzed by *Aspergillus oryzae* β-galactosidase was performed in the presence of aqueous-organic solvent mixtures. By using TLC analysis, an oligosaccharide product (Oligo-I, Rf 0.19) was detected when using citrate buffer containing triethyl phosphate (TEP) or methoxyethyl acetate (MEA), while no product was observed with 1,4-butanediol, 1,5-pentanediol or acetonitrile (all solvents were 50% v/v mixed with citrate buffer). When the amount of oligosaccharide was determined by HPLC, synthesis in TEP solution gave 1.6 and 6.8 times higher product yield than in buffer alone and MEA solution, respectively. However, yield obtained from synthesis by soluble (non-lyophilized) enzyme in TEP solution was only 18.5%. To overcome low enzyme activity under co-solvent condition, co-lyophilization of the enzyme from citrate buffer with the addition of cyclodextrin (CD), a group of macrocyclic excipients, was investigated. Comparing with its hydroxypropyl and methyl derivatives, β-CD worked best. β-galactosidase: β-CD at 1:5 w/w increased oligosaccharide synthesis in 50% TEP by 20.9% compared to synthesis from enzyme lyophilized in the absence of CD or non-lyophilized enzyme. The optimal condition for oligosaccharide synthesis in 50% TEP using the β-galactosidase-β-CD co-lyophilizate was 0.3 M lactose incubated with 0.025 mg enzyme at pH 4.5, 40 °C for 36-72 hours. In addition to the major Oligo-I product, a minor Oligo-II (Rf 0.10) product was also observed. Yield of Oligo-I after optimization was 31.6%, an increase over the non-optimized condition in the absence of β-CD of 63.6%. Thus β-CD could significantly enhance oligosaccharide synthesis catalyzed by β-galactosidase in TEP co-solvent system. When oligosaccharide products were analyzed by MS and NMR techniques, Oligo-I is characterized as *O*-β-D-galactopyranosyl-(1-6)-*O*-β-D-galactopyranosyl-(1-4)-D-glucopyranose or 6'-galactosyl-lactose, and Oligo-II is a tetrasaccharide mixed with a pentasaccharide.

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Academic year 2006

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my advisor, Associate Professor Piamsook Pongsawasdi, for her excellent instruction, guidance, encouragement, attention and support throughout this thesis.

My gratitude is also extended to Associate Professor Aran Incharoensakdi, Assistant Professor Kanoktip Packdibamrung and Dr. Manchumas Prousoontorn for their valuable suggestion and comments and also dedicating valuable time for thesis examination.

My acknowledgement is expressed to Assistant Professor Warinthorn Chavasiri for guidance and helpfulness in techniques and useful suggestions.

My appreciation is also expressed to all staff members and all friends for their kind assistance, friendship and helpfulness especially Mr. Surachai Yaiyen, Mr. Wanchai Yenpetch and Mr. Pisanu Thanadolsathien, at Department of Biochemistry, Faculty of Science.

Finally, the greatest gratitude is expressed to my parents and my family for their infinite love, encouragement, understanding and everything giving to my life.

This work was financial supported from Ratchadaphiseksomphot Endowment Fund to Starch and Cyclodextrin Research Unit and Graduate Thesis Grant from Graduate School, Chulalongkorn University.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CONTENTS

	Page
THAI ABSTRACT	iv
ENGLISH ABSTRACT	v
ACKNOWLEDGEMENTS	vi
CONTENTS	vii
LIST OF TABLES	xi
LIST OF FIGURES	xii
ABBREVIATIONS	xiv
CHAPTER I : INTRODUCTION	1
1.1 Oligosaccharides	1
1.1.1 Chemical composition of oligosaccharides	1
1.1.2 Properties of oligosaccharides	2
1.2 Prebiotics	4
1.2.1 The non-digestible oligosaccharides	5
1.2.2 Prebiotic safety status	5
1.3 Oligosaccharide production	7
1.3.1 Chemical production	7
1.3.2 Enzymatic production	7
1.3.2.1 Glycosyltransferase	9
1.3.2.2 Glycosidase	9
1.3.2.2.1 Equilibrium-controlled synthesis	10
1.3.2.2.2 Kinetically-controlled synthesis	10
1.3.3 Current commercial oligosaccharide production process	11
1.4 β-galactosidase	13
1.5 Cyclodextrin	14
1.5.1 Cyclodextrin and inclusion complex	14
1.5.2 Applications of cyclodextrin	19
1.5.3 β-cyclodextrin safety status	20
1.6 β-galactosidase and cyclodextrin	22

CHAPTER II : MATERIALS AND METHODS 23
2.1 Equipments 23
2.2 Chemicals 23
2.3 Protein determination 25
2.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) 25
2.4.1 Coomassie blue staining 25
2.5 Enzyme assay 25
2.5.1 Hydrolysis activity 25
2.5.2 Oligosaccharide synthetic activity 26
2.6 Oligosaccharide determination 26
2.6.1 Thin layer chromatography (TLC) 26
2.6.2 High performance liquid chromatography (HPLC) 27
2.7 Co-lyophilization of β-galactosidase with cyclodextrin or its derivatives 27
2.7.1 Effect of co-lyophilization of β-galactosidase with cyclodextrin or its derivatives on oligosaccharide synthesis 27
2.7.2 Effect of ratio of β-galactosidase to cyclodextrin co-lyophilized on oligosaccharide synthesis 28
2.8 Optimization of oligosaccharide synthesis 28
2.8.1 Effect of lactose 28
2.8.2 Effect of enzyme 28
2.8.3 Effect of pH 29
2.8.4 Effect of temperature 29
2.8.5 Effect of reaction time 29
2.9 Measurement of fluorescence spectrum of β-galactosidase/cyclodextrin complex 29
2.10 Fourier transform infrared (FT-IR) spectroscopy 30
2.11 Preparative layer plate (PLC) 30
2.12 Mass spectrometry (MS) 30
2.13 Nuclear magnetic resonance (NMR) 31

CHAPTER III : RESULTS 32
3.1 Purity of β-galactosidase 32
3.2 Selection of most appropriate organic solvent 32
3.2.1 Determination of hydrolysis activity of β-galactosidase 32
3.2.2 Determination of oligosaccharide synthetic activity of β-galactosidase 35
3.2.3 Determination of hydrolysis activity of lyophilized β-galactosidase 40
3.2.4 Determination of oligosaccharide synthetic activity of lyophilized β-galactosidase 40
3.3 Effect of cyclodextrins on oligosaccharide synthesis by β-galactosidase in the presence of organic solvent 43
3.3.1 Effect of type of cyclodextrins 43
3.3.2 Effect of ratio of β-galactosidase to β-cyclodextrin 46
3.4 Optimization of oligosaccharide synthesis 46
3.4.1 Effect of lactose concentration 49
3.4.2 Effect of amount of enzyme 49
3.4.3 Effect of pH 52
3.4.4 Effect of temperature 52
3.4.5 Effect of reaction time 56
3.5 Measurement of fluorescence spectrum of β-galactosidase-β-cyclodextrin co-lyophilizate 56
3.6 Fourier transform infrared (FT-IR) spectroscopy 59
3.7 Characterization of oligosaccharide products 59
3.7.1 Mass spectrometry (MS) 59
3.7.2 Nuclear magnetic resonance (NMR) 63

CHAPTER IV : DISCUSSION 68
4.1 Selection of most appropriate organic solvent for synthetic reaction of β-galactosidase 68

4.2 Effect of cyclodextrins on oligosaccharide synthesis by β-galactosidase in the presence of organic solvent 72

4.3 Optimization of oligosaccharide synthesis 74

4.4 Investigation of the change in secondary and tertiary structures of β-galactosidase upon co-lyophilization with β-cyclodextrin 75

4.5 Characterization of oligosaccharide products 76

CHAPTER V : CONCLUSIONS 78

REFERENCES 79

APPENDICES 86

BIOGRAPHY 94

LIST OF TABLES

Table **Pages**

1. Various kinds of oligosaccharides 3
2. Properties of oligosaccharides 3
3. Non-digestible oligosaccharides with bifidogenic functions commercially available 7
4. Physical properties of α-, β- and γ-cyclodextrin 16
5. Possible effects of the formation of inclusion complexes on properties of guest molecules 18
6. Regulatory status of the natural cyclodextrins (January 2004) 22
7. ^{1}H-NMR chemical shifts for Oligo-I (Gal''-Gal'-Glu) 65
8. ^{13}C-NMR chemical shifts for Oligo-I (Gal''-Gal'-Glu) 67

LIST OF FIGURES

Figure	Pages
1. Typical chemical synthesis of a disaccharide	9
2. Schematic representation of production processes of non-digestible oligosaccharides	12
3. Chemical structure of α-, β- and γ-cyclodextrin	16
4. Dimension and hydrophilic/hydrophobic regions of the CD molecules	17
5. Schematic representation of CD inclusion complex formation	18
6. SDS-PAGE of β-galactosidase	33
7. Hydrolysis activity of β-galactosidase in organic solvent mixtures	34
8. TLC chromatogram of synthetic reaction products of β-galactosidase in co-solvents	36
9. TLC chromatogram of synthetic reaction products of β-galactosidase in triethyl phosphate and methoxyethyl acetate co-solvent	37
10. HPLC chromatograms of synthetic reaction products of β-galactosidase in 50%TEP co-solvent at incubation time of A) 0 hour; B) 48 hours	38
11. Oligosaccharide synthesis of β-galactosidase in different solvent	39
12. Hydrolysis activity of lyophilized β-galactosidase in organic solvent mixtures	41
13. Oligosaccharide synthesis of β-galactosidase compared between non-lyophilized and lyophilized enzyme	42
14. TLC chromatogram of synthetic reaction products of β-galactosidase lyophilized with Cyclodextrin and linear oligosaccharides	44
15. Oligosaccharide synthesis of β-galactosidase lyophilized with cyclodextrin and linear oligosaccharides. Synthesis was in A) buffer; B) 50%TEP co-solvent	45

16. TLC chromatogram of synthetic reaction products of β-galactosidase co-lyophilized with various ratios of β-cyclodextrin 47
17. Oligosaccharide synthesis by various ratios of β-galactosidase-β-cyclodextrin co-lyophilizate 48
18. TLC Chromatogram of synthesized oligosaccharides produced in various concentrations of lactose 50
19. Effect of lactose concentration on oligosaccharide synthesis 50
20. TLC chromatogram of synthesized oligosaccharides produced in various amounts of enzyme 51
21. Effect of amount of enzyme on oligosaccharide synthesis 51
22. TLC chromatogram of synthesized oligosaccharides produced at various pHs 53
23. Effect of pH on oligosaccharide synthesis 54
24. TLC chromatogram of synthesized oligosaccharides produced at various temperatures 55
25. Effect of temperature on oligosaccharide synthesis 55
26. TLC Chromatogram of synthesized oligosaccharides produced at different reaction time 57
27. Effect of reaction time on oligosaccharide synthesis 57
28. Fluorescence emission spectrum of β-galactosidase in 50% TEP (v/v) 58
29. FT-IR spectrum of β-galactosidase 60
30. ESI-TOF mass spectra of Oligo-I 61
31. ESI-TOF mass spectra of Oligo-II 62
32. The 500 MHz ^{1}H-NMR spectra of Oligo-I 64
33. The 125 MHz ^{13}C-NMR spectra of Oligo-I 66

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

ABBREVIATIONS

A	absorbance
°A	angstrom
bw	body weight
BSA	bovine serum albumin
°C	degree Celsius
CDs	cyclodextrin
CFU	colony-forming unit
^{13}C-NMR	Carbon-13 nuclear magnetic resonance
DP	degree of polymerization
ESI-TOF-MS	Electrospray Ionization-Time of Flight Mass Spectrometry
FT-IR	Fourier Transform Infrared Spectroscopy
FT-NMR	Fourier Transform Nuclear Magnetic Resonance
g	gram
GOS	galacto-oligosaccharide
^{1}H-NMR	Proton nuclear magnetic resonance
HPβCD	hydroxypropyl-β-cyclodextrin
hr	hour
l	litre
M	molar
MβCD	methyl-β-cyclodextrin
mg	milligram
min	minute
mM	millimolar
NDOs	the non digestible oligosaccharides
NOEL	no-observed-effect level
PLC	preparative layer plate
SCFA	short chain fatty acid
t	ton
μl	microlitre

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER I

INTRODUCTION

1.1 Oligosaccharides

1.1.1 Chemical composition of oligosaccharides

Oligosaccharides consist of short chains of monosaccharide units joined by characteristic linkage called glycosidic bonds. Typical is 2-20 units (Nelson and Cox, 2000). Monosaccharides have multiple hydroxyl groups, thus various glycosidic linkages are possible. Indeed, the wide array of these linkages in concert with the wide variety of monosaccharides and their many isomeric forms makes complex carbohydrates information-rich molecules (Berg et al., 2002).

Various types of oligosaccharides have been found as natural components in many common foods including fruits, vegetables, milk and honey. (Nakakuki, 2002) Three abundant disaccharides are sucrose, lactose and maltose. Sucrose (common table sugar) is obtained commercially from cane or beet. The anomeric carbon atoms of a glucose unit and a fructose unit are joined in this disaccharide; the configuration of this glycosidic linkage is α for glucose and β for fructose. Lactose, the disaccharide of milk, consists of galactose joined to glucose by a β-1,4-glycosidic linkage. In maltose, two glucose units are joined by an α-1,4-glycosidic linkage. (Berg et al., 2002)

Several oligosaccharides such as starch-related, sucrose-related and lactose-related oligosaccharides have been developed, as shown in Table 1. Also, xylo-oligosaccharides, manno-oligosaccharides and chitin/chitosan oligosaccharides have been produced from various polysaccharides such as xylan, agar, mannan, chitin and chitosan as the raw materials. At present, these oligosaccharides have been widely utilized in foods, beverages and confectionery processing applying these various properties of oligosaccharides.

1.1.2 Properties of oligosaccharides

Oligosaccharides are functional food ingredients that have a great potential to improve the quality of many foods. In addition to providing useful modifications to physicochemical properties of foods, it has been reported that these oligosaccharides have various physicological functions such as the improvement of intestinal microflora based on the selective proliferation of bifidobacteria, stimulation of mineral absorption, anticariogenicity, and the improvement of both plasma cholesterol and blood glucose level. (Nakakuki, 2002) Functional properties evaluated until now are summarized in Table 2.

Oligosaccharides are water soluble and typically 0.3–0.6 times as sweet as sucrose. In fact, the sweetness depends on chemical structure, the degree of polymerization and the levels of mono- and disaccharides in the mixture (Crittenden and Playne, 1996; Voragen, 1998). The sweetness decreases with the longer oligosaccharide chain length. This low sweetness intensity is quite useful in the various kinds of foods where the use of sucrose is restricted by its high sweetness property (Roberfroid and Slavin, 2000). They may be used as bulking agents in conjunction with artificial sweeteners such as aspartame or sucralose, for example, with the advantage to mask the aftertastes produced by some of these intense sweeteners. In addition, when compared with mono- and disaccharides, the higher molecular weight oligosaccharide provides increased viscosity, leading to improved body and mouthfeel (Crittenden and Playne, 1996).

The stability can greatly differ for the various classes of oligosaccharides depending on the sugar residues present, their ring form and anomeric configuration and linkage types. Generally β-linkages are stronger than α-linkages, and hexoses are more strongly linked than pentoses. Nevertheless, as a whole, at $pH < 4.0$ and treatments at elevated temperatures or prolonged storage at room conditions, oligosaccharides present in food can be hydrolyzed resulting in loss of nutritional and physicochemical properties (Voragen, 1998). The oligosaccharides can also be used to alter the freezing temperature of frozen foods, and to control the intensity of browning due to Maillard reactions in heat-processed foods. They also provide a high moisture-retaining capacity, preventing excessive drying, and a low water activity, which is convenient in controlling microbial contamination (Crittenden and Playne, 1996).

Table 1 Various kinds of oligosaccharides (Nakakuki, 2002)

Raw material	Product
Starch	Malto-oligosaccharides: maltose ~ maltoheptaose Isomalto-oligosaccharides: isomaltose, panose, isomaltotriose Cyclodextrins (CDs): α-CD, β-CD, γ-CD, HP-β-CD, branched CDs Others: maltitol, gentio-oligosaccharides, trehalose, nigerose
Sucrose	Glycosylsucrose, fructo-oligosaccharides, palatinose (isomaltulose), lactosucrose, xylosucrose, raffinose, stachyose, trehalulose
Lactose	Galacto-oligosaccharides, lactosucrose, lactulose, lactitol
Xylan, agar, mannan, chitin, chitosan, etc.	Xylo-oligosaccharides, agaro-oligosaccharides, manno-oligosaccharides, chitin/chitosan oligosaccharides, etc.

Table 2 Properties of oligosaccharides (Nakakuki, 2002)

Physicochemical property	Sweetness, bitterness, hygroscopicity, water activity, reinforcement agent for drinks, stabilization of active substances (protein, flavor, color, etc.), inclusion capability, etc.
Biological property	Digestibility, nondigestibility, noncariogenicity, anticariogenicity, bacteriostatic action, selective proliferation of bifidobacteria, improvement of serum lipids, and blood glucose, etc.
Other properties	Specific substrate for enzyme, enzyme inhibitors, elicitors, etc.

Most oligosaccharides are quantitatively hydrolyzed in the upper part of the gastrointestinal tract. The resulting monosaccharides are transported via the portal blood to the liver and, subsequently, to the systemic circulation. Such carbohydrates are essential for health as they serve both as substrates and regulators of major metabolic pathways. Nevertheless, some oligosaccharides present specific physicochemical properties and resist to the digestive process, reaching the caeco-colon as they have been eaten. In the caeco-colon, most (but not necessarily all) of the non-digestible oligosaccharides are hydrolyzed to small oligomers and monomers, which are further metabolized by one, a few, or most of the anaerobic bacteria. Such a metabolic process, know as fermentation, not only serves the bacteria by providing energy for proliferation, but it also produces gases (H_2, CO_2, CH_4), which are metabolically useless to the host, and small organic acids (short-chain fatty acids – SCFA) such as acetate, propionate, butyrate and L-lactate. Even though they do not provide the body with monosaccharides, the non-digestible oligosaccharides are indirect energy substrates and metabolic regulators (Delzenne and Roberfroid, 1994).

1.2 Prebiotics

"Prebiotic" is a dietary ingredient that reaches the large intestine in an intact form and has a specific metabolism directed towards advantageous rather than adverse bacteria. This would ultimately lead to a marked change in the gut microflora composition. The small intestine has a larger bacterial load that consists of facultative anaerobes such as lactobacilli, streptococci, and enterobacteria as well as anaerobes such as *Bifidobacterium* spp., and clostridia at level ~10^4-10^8 CFU/ml. The most heavily colonized region, however, is the colon, with a total population of 10^{11}-10^{12} CFU/ml of contents (Cummings et al., 1989). Consisting of higher levels of obligate anaerobes and lower levels of facultative aerobes (Rastall, 2004), the colonic microflora is very complex.

In terms of health, the most significant organisms are believed to be the bifidobacteria (Gibson and Roberfroid, 1995). Bifidobacteria are the major component of the microbial barrier to infection. Bifidobacteria produce a range of antimicrobial agents that are active against Gram-positive and -negative organisms. Lactobacilli are also health positive and produce a range of antimicrobial agents, but they are present in much lower levels in the human colon. In addition to producing

antimicrobial agents, a large population of beneficial bacteria competitively excludes pathogens by occupying receptor sites and competing for space, nutrients, etc (Rastall, 2004).

Prebiotic fermentation should be directed towards potentially health promoting bacteria, with indigenous lactobacilli and bifidobacteria currently being the preferred target organisms. The approach is therefore similar to that dietary fiber, but with prebiotics acting in a much more tailored manner, in that the bacterial fermentation/growth is selective. The most efficient prebiotics will also reduce or suppress numbers and activities of organisms seen as pathogenic.

Moreover, and because of the intestinal fermentation, prebiotics may have certain physiological effects including: control of transit time and mucosal motility, regulation of epithelial cell proliferation, influence on nutrient (in particular ion) bioavailability, modulate immune activity and affect endocrine function. There may also be certain systemic influences such as carbohydrate or lipid homeostasis.

1.2.1 The non-digestible oligosaccharides

At present, food component which seems to exert the best prebiotic effect are the non digestible oligosaccharides (NDOs). These are oligomeric carbohydrates, the osidic bond of which is in a spatial configuration that allows resistance to hydrolytic acitivities of intestinal digestive enzyme, but are sensitive to metabolic effects of colonic bacteria. These microorganisms can then ferment the carbohydrate to produce short chain carboxylic acids and gases, as well as increase metabolic energy, growth and proliferation (Roberfroid, 1997). The carbohydrates contain mixtures of oligomers of different chain lengths and are characterized by their average degree of polymerization (DP) (Cummings and Roberfroid, 1997). NDOs that present bifidogenic function, and are commercially produced were grouped into 13 classes (Table 3) (Sako et al., 1999).

1.2.2 Prebiotic safety status

The evaluation of an acceptable dose is difficult because each individual has his own feeling about acceptable and non-acceptable intestinal discomfort. However, excessive consumption doses of prebiotics may cause intestinal discomfort, flatulence

Table 3 Non-digestible oligosaccharides with bifidogenic functions commercially available (slightly modified from Crittenden and Playne, 1996 and Sako et al., 1999)

Class of oligosaccharide	**Estimated[a] production in 1995 (ton)**	**Molecular structure[b]**
Cyclodextrins	4,000	$(Gu)_n$
Fructo-oligosaccharides	12,000	$(Fr)_n$-Gu
Galacto-oligosaccharides	15,000	$(Ga)_n$-Gu
Gentio-oligosaccharides	400	$(Gu)_n$
Glycosylsucrose	4,000	$(Gu)_n$-Fr
Isomalto-oligosaccharides	11,000	$(Gu)_n$
Isomaltulose (or palatinose)	5,000	$(Gu\text{-}Fr)_n$
Lactosucrose	1,600	Ga-Gu-Fr
Lactulose	20,000	Ga-Fr
Malto-oligosaccharides	10,000	$(Gu)_n$
Raffinose	*	Ga-Gu-Fr
Soybean oligosaccharides	2,000	$(Ga)_n$-Gu-Fr
Xylo-oligosaccharides	300	$(Xy)_n$

*** no data.**

[a] Data were obtained by surveying major manufacture of food-grade NDOs.

[b] Ga, Galactose; Gu, Glucose; Fr, Fructose; Xy, Xylose.

or even diarrhea because of their osmotic effect, which may transfer water into the large bowel (an effect which is inversely related to chain length), and because of their high fermentation rate and production of gases (Roberfroid and Slavin, 2000). For example, galacto-oligosaccharides consumption higher than 20 g/day, and fructo-oligosaccharides consumption higher than 40 g/day are reported to cause diarrhea (Sako et al., 1999).

1.3 Oligosaccharide production

1.3.1 Chemical production

Chemical methods for the synthesis of oligosaccharides are well developed (Paulsen, 1984; Schmidt, 1986). However, carbohydrates contain multiple hydroxyl groups of similar reactivity and chemical methods are complicated by the many protection steps that are necessary for regioselective synthesis (Figure 1).

The number of steps increases with the size of oligosaccharide, so that, while synthesis of a disaccharide may require five to seven steps, a trisaccharide may require more than ten steps. Total yields are often low and large-scale synthesis is not practical. In addition, stereospecific reactions giving the correct anomer (α or β) are often difficult (Nilsson, 1988).

1.3.2 Enzymatic production

The use of enzymes in synthesis of complex carbohydrates offers several advantages over chemical methods. A wide variety of regiospecific and often highly regioselective reactions can be catalysed very efficiently without protection of the hydroxyl groups; these take place under mild conditions, often at room temperature and close to neutral pH, and organic solvents and hazardous chemicals or catalysts can be avoided. Immobilization of the enzyme allows reuse while production of the catalyst by fermentation facilitates large scale synthesis. Two types of enzyme have been used for preparation of complex oligosaccharides, the glycosyltransferase (EC 2.4) and the glycosidase (EC 3.2) (Nilsson, 1988).

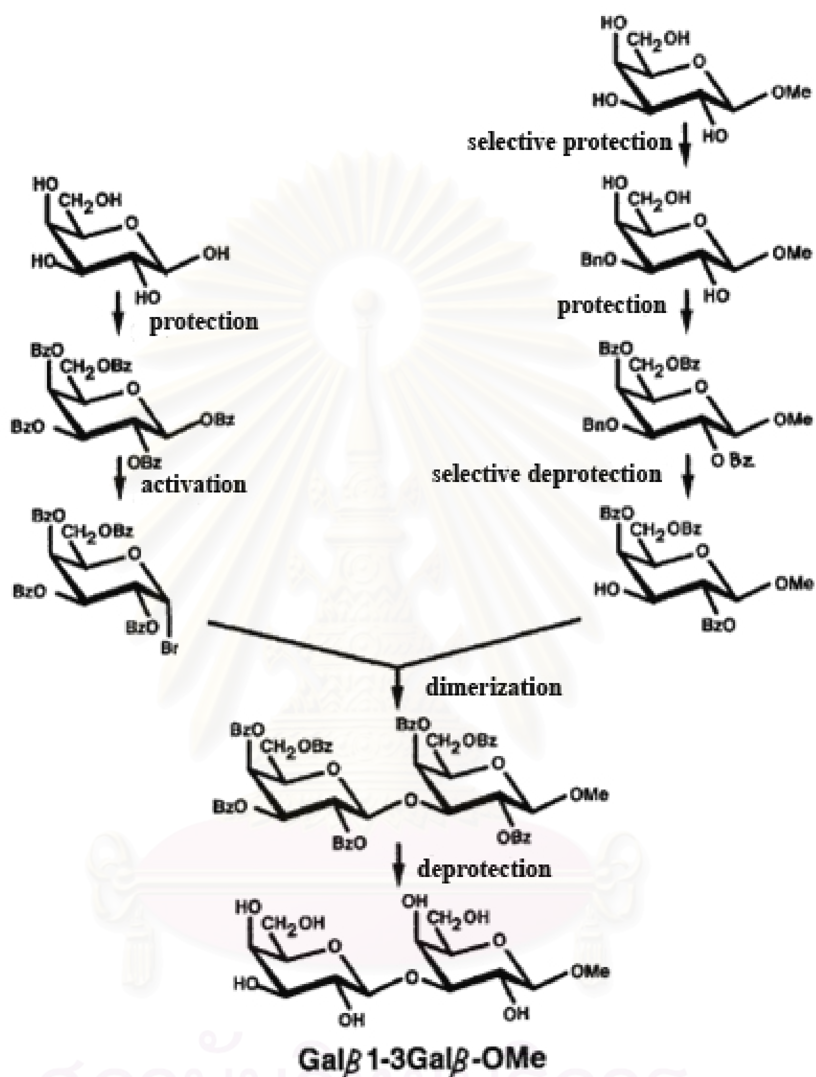
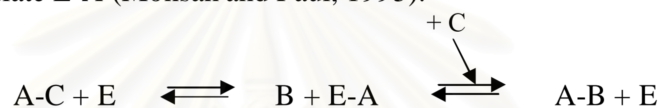


Figure 1 Typical chemical synthesis of a disaccharide (Nilsson, 1988)

1.3.2.1 Glycosyltransferase

Glycosyltransferase (EC 2.4) catalyzed the stereo- and regiospecific transfer of a monosaccharide from a donor substrate (glycosyl nucleotide) to an acceptor substrate. This method has the advantage of high efficiency and selectivity. Its major drawbacks are the requirement for a complex glycosyl donor and the relative inaccessibility of the glycosyltransferases; moreover, this method requires nucleotide sugar which is costly. Long chain oligosaccharides are usually produced by this reaction (Nilsson, 1988; Crout and Vic, 1998). The energy necessary for the synthesis reaction is provided by the original osidic bond and stored as a covalent glycosyl-enzyme intermediate E-A (Monsan and Paul, 1995).



A-B, donor carbohydrate or glycoside; E, glycosyltransferase; C, acceptor

Some glycosyltransferases such as cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) does not require nucleotide sugar for activitation of donor substrate (Maitin et al., 2004). Its use for industrial oligosaccharide synthesis becomes real after the enzyme has been commercially produced.

1.3.2.2 Glycosidase

Glycosidase (glycosylhydrolase) (EC 3.2), the enzyme is responsible for the hydrolytic cleavage of glycosidic bonds in nature. The glycosidase, in general, can be divided into two groups: the exoglycosidases, which cleave glycosidic bonds at the nonreducing end of the oligosaccharides, and the endoglycosidases, which cleave internal glycosidic bonds. The advantage of utilizing glycosidase is that using relative simple glycosyl donors and generally more available and less expensive. Short chain oligosaccharides are generally produced by this enzyme (Nilsson, 1988; Ichikawa et al., 1992; Crout and Vic, 1998).

Glycosidase is a hydrolytic enzyme and synthesis is carried out either as an equilibrium-controlled or as a kinetically controlled process. The hydrolytic activity of glycosidase can also be used for preparation of oligosaccharides from larger structure.

1.3.2.2.1 Equilibrium-controlled synthesis

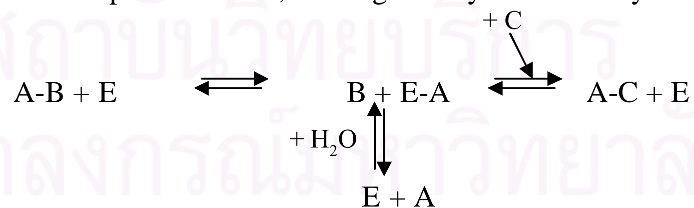
Equilibrium-controlled synthesis is reversion of the hydrolytic reaction (reverse hydrolysis reaction). In reverse hydrolysis, sometimes called the thermodynamic approach, a high concentration of reactant, high temperature reaction, low water activity and long incubation time are used to drive the reaction towards equilibrium (Nilsson, 1988; Monsan and Paul, 1995; Crout and Vic, 1998). It constitutes a very cost-effective and simple procedure making it very attractive for industrial scale-up (Crout and Vic, 1998).

where A and B are glycosyl moieties

Glycosidases are widely available and can be highly thermostable. This allows operating at high temperature. The main limits of the reverse hydrolysis approach are the low activity of glycosidase in concentrated carbohydrate solutions, the low yield of carbohydrate conversion and the production of isomer mixture (Monsan and Paul, 1995).

1.3.2.2.2 Kinetically-controlled synthesis

Kinetically controlled synthesis (transglycosylation) uses glycosidase enzyme to catalyze the transfer of a glycosyl moiety from a glycoside derivative onto an acceptor molecule, which generally contains a hydroxyl group:



A-B, glycoside donor, E, Glycosidase; C, acceptor.

In this case, the reaction efficiency is kinetically controlled, as the product, A-C, is a potential substrate of the glycosidase enzyme (Monsan and Paul, 1995). However, the donor glycoside is consumed during reaction and its reuse

is not possible. Hydrolysis competes and the maximum yield depends on the rate of product formation relative to the rate of hydrolysis (Nilsson, 1988).

1.3.3 Current commercial oligosaccharide production process

Industrial production processes have been established to extract the NDOs from natural sources, by hydrolyzing polysaccharides, and by enzymatic or chemical synthesis from disaccharide substrates. With the exception of soybean oligosaccharides and raffinose (which are produced by direct extraction) and lactulose (which is produced by isomerization reaction), the NDOs are manufactured using enzymatic processes. They are either “built up” from simple sugars, such as sucrose or lactose, by enzymatic transglycosylation reactions, or formed by controlled enzymatic hydrolysis of polysaccharides, such as starch or xylan (Figure 2) (Sako et al., 1999). Typically, cheap oligosaccharides are utilized as donors, such as sucrose, lactose and starch-derived oligomers. These processes usually produce a range of oligosaccharides differing in their degree of polymerization and sometimes in the position of the glycosidic linkages. Unreacted substrates and monosaccharides are also present after oligosaccharide formation. Such contaminating sugars are often removed by membrane or chromatographic procedures to form higher-grade products that contain purer oligosaccharides (Crittenden and Playne, 1996).

Some NDOs are produced from two raw materials. For instance, lactosucrose is produced using lactose and sucrose, and glycosylsucrose is produced using sucrose and liquid starch.

Galacto-oligosaccharides are commercially produced from lactose by the action of β-galactosidase, which have transgalactosylation activity. In the industrial production process of galacto-oligosaccharides, a highly concentrated solution of lactose, which is usually purified from cow's milk whey, is used as substrate solution in this reaction (Sako et al., 1999).

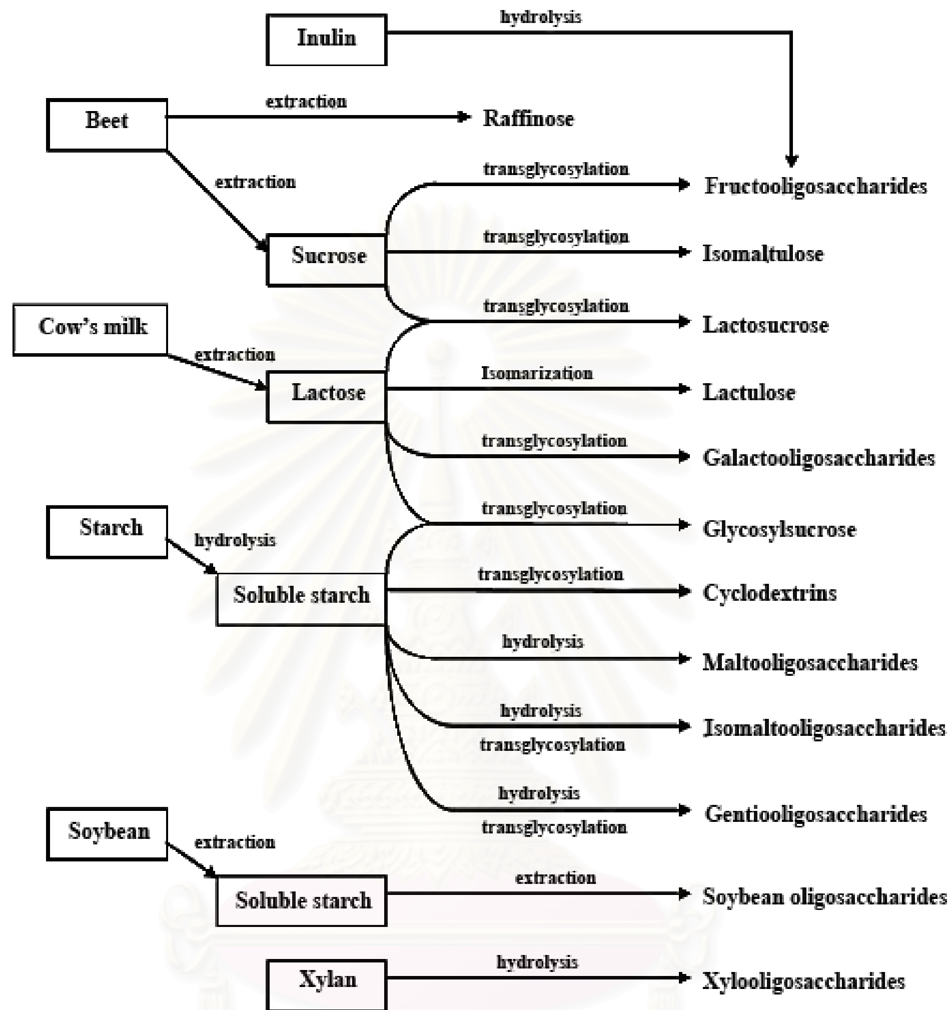


Figure 2 **Schematic representation of production processes of non-digestible oligosaccharides (Sako et al., 1999).**

In general, food grade oligosaccharides are not pure products, but are mixtures containing oligosaccharides of different degrees of polymerization, the original polysaccharide or disaccharide, and monomeric sugars. Most manufacturers produce several classes of products; higher grades contain purer oligosaccharide mixtures with lower levels of contaminating monosaccharides and reactant di- or polysaccharides. For NDOs, the absence of simple sugars lowers cariogenicity and calorific value, and allows the oligosaccharides to be included in diabetic foods (Crittenden and Playne, 1996).

1.4 β-galactosidase

β-galactosidase (β-D-galactoside galactohydrolase, lactase, EC 3.2.1.23) is one enzyme in the hydrolase group. It catalyzes hydrolysis of lactose (Huber et al., 1976). β-galactosidase is widely distributed in microorganisms, animals and plants. This enzyme can synthesize oligosaccharides by either equilibrium controlled or kinetically controlled synthesis (Iwasaki et al., 1996; Giacomini et al., 2002). β-galactosidase has been used in commercial production of oligosaccharide products such as galacto-oligosaccharides, lactulose. The linkage between the galactose units depends on source of enzyme, such as β-1,4 bond is the main product when β-galactosidase from *Bacillus circulans* or *Cryptococcus laurentii* is used, while β-1,6 bond is obtained when β-galactosidase is derived from *Aspergillus oryzae* or *Streptococcus thermophilus* (Reuter et al., 1999; Sako et al., 1999). In 1996, Iwasaki and colleagues used the equilibrium approach to synthesize galacto-oligosaccharides from lactose by an enzymic batch reaction using β-galactosidase of *Aspergillus oryzae*. A maximum oligosaccharide conversion of 30% was obtained with lactose feed concentration greater than 1.11 M. By NMR analysis, the configuration and linkage of the main galacto-oligosaccharide product was characterized as β-1,6 linkage (Chen et al., 2001). In the production process catalyzed by β-galactosidase, higher yield of oligosaccharides can be obtained by optimizing condition for improvement of enzyme activity (Iwasaki et al., 1996), immobilizing enzyme in reverse micelles (Chen et al., 2001), or in celite or chitosan (Gaur et al., 2006), or using organic solvent system to reduce water content and improve reverse hydrolysis reaction (Sauerbrei and Thiem, 1992; Nilsson, 1989; Vocadlo and Withers, 2000).

In 1989, Nilsson reported using glycosidase and β-D-galactoside 3-α-sialyl-transferase in 23% dimethylformamide for producing many kinds of di- and tri-saccharides. In 1992, Sauerbrei and Thiem produced β-Glc(1-2)β-Glc-$PhNO_2$-*p* and α-Glc(1-4)β-Glc-$PhNO_2$-*p* in 50% acetonitrile by β-galactosidase from *Aspergillus oryzae*. The major limitation of reaction in organic solvent system is reduction or loss of enzyme activity when compared with reaction in aqueous buffer solution (Klibanov, 1997; Triantafyllou et al., 1997)

1.5 Cyclodextrins

1.5.1 Cyclodextrin and inclusion complex

Cyclodextrins (Schardinger dextrins, Cycloamylose, Cyclomaltose or Cycloglucans) are the oligomers of anhydroglucose units join to form a ring structure with α-1,4 glycosidic bonds. The main CDs synthesized naturally by the cyclodextrin glycosyltransferase (CGTase) are composed of 6, 7 and 8 glucose units called α, β, and γ- cyclodextrin, respectively (Figure 3) (Szejtli, 2004). They have different physical properties as summarized in Table 4 (Szejtli, 1988, cited in Szejtli, 1998)

As a consequence of the $^4C_1$ conformation of the glucopyranose units, all secondary hydroxyl groups are situated on one of the two edges of the ring, whereas all the primary ones are placed on the other edge. The ring, in reality, is a conical cylinder, which is frequently characterized as a doughnut or wreath-shaped truncated cone. The cavity is lined by the hydrogen atoms and the glycosidic oxygen bridges, respectively. The nonbonding electron pairs of the glycosidic-oxygen bridges are directed toward the inside of the cavity, producing a high electron density there and lending to it some Lewis-base character.

The C-2-OH group of one glucopyranoside unit can form a hydrogen bond with the C-3-OH group of the adjacent glucopyranose unit. In the β-CD molecule, a complete secondary belt is formed by these H bonds; therefore, the β-CD is a rather rigid structure. This intramolecular H-bond formation is probably the explanation for the observation that β-CD has the lowest water solubility of all CDs.

The H-bond belt is incomplete in the α-CD molecule, because one glucopyranose unit is in a distorted position. Consequently, instead of the six possible H bonds, only four can be established simultaneously. The γ-CD is a non-coplanar,

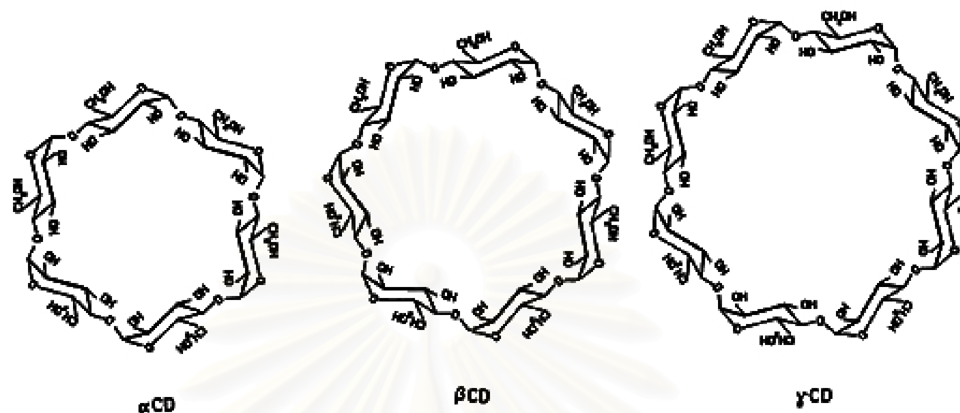


Figure 3 **Chemical structure of α-, β- and γ-cyclodextrin (Szejtli, 2004)**

Table 4 **Physical properties of α-, β- and γ-cyclodextrin (Szejtli, 1998)**

	α	**β**	**γ**
Glucose monomers	6	7	8
Molecular weight	972	1135	1297
Water solubility (g/100 ml: 25 °C)	14.5	1.85	23.2
Cavity diameter (°A)	4.7-5.3	6.0-6.5	7.5-8.3
Height of torus (°A)	7.9±0.1	7.9±0.1	7.9±0.1
Diameter of outher periphery (°A)	14.6±0.4	15.4±0.4	17.5±0.4
Approx volume of cavity (°A^3)	174	262	427
Water of crystallization (wt%)	10.2	13.2-14.5	8.13-17.7

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more flexible structure; therefore, it is most soluble of the three CDs. Figure 4 shows a sketch of the characteristic structural features of CDs. On the side where the secondary hydroxyl groups are situated, the cavity is wider than on the other side where free rotation of the primary hydroxyls reduces the effective diameter of the cavity (Szejtli, 2004).

In an aqueous solution, the slightly apolar cyclodextrin cavity is occupied by water molecules that are energetically unfavored (polar–apolar interaction), and therefore can be readily substituted by appropriate "guest molecules", which are less polar than water (Figure 5). The dissolved cyclodextrin is the "host" molecule, and part of the "driving force" of the complex formation is the substitution of the high-enthalpy water molecules by an appropriate "guest" molecule. This is the essence of "molecular encapsulation". One, two, or three CD molecules contain one or more entrapped "guest" molecules. Most frequently the host : guest ratio is 1:1. However 2:1, 1:2, 2:2, or even more complicated associations, and higher order equilibria exist, are almost always simultaneously. The formed inclusion complexes can be isolated as stable crystalline substances. Upon dissolving these complexes, an equilibrium is established between dissociated and associated species, and this is expressed by the complex stability constant Ka. The association of the CD and guest molecules and the dissociation of the formed CD/guest complex are governed by a thermodynamic equilibrium (Szejtli, 1998).

Cyclodextrins are used to obtain certain benefits that result from complexation with the cyclodextrins. These include alteration of the solubility of the guest compound, stabilization against the effects of light, heat, and oxidation, masking of unwanted physiological effects, reduction of volatility, and others (van der Veen, 2000) (Table 5).

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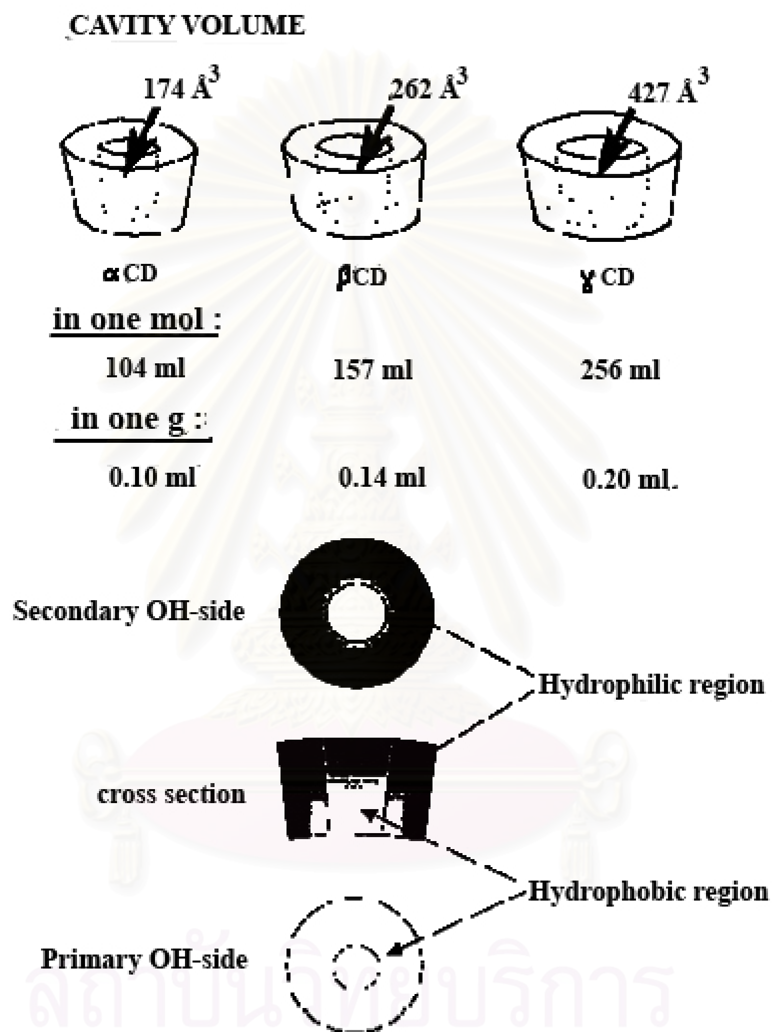

Figure 4 Dimension and hydrophilic/hydrophobic regions of the CD molecules (Szejtli, 2004)

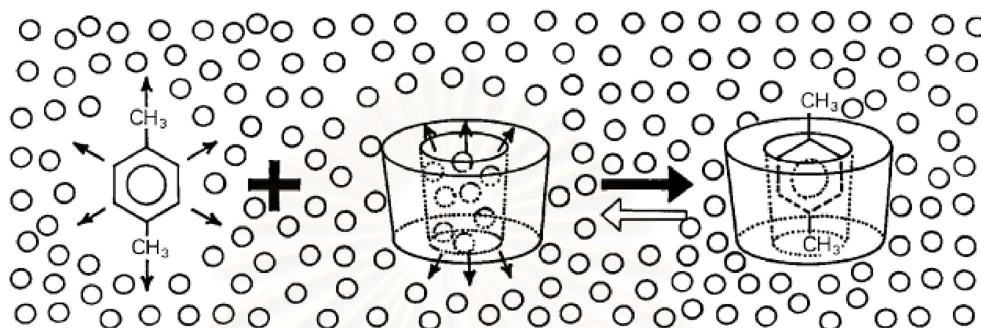

Figure 5 **Schematic representation of CD inclusion complex formation. *p*-Xylene is the guest molecule; the small circles represent the water molecules (Szejtli, 1998)**

Table 5 **Possible effects of the formation of inclusion complexes on properties of guest molecules (van der Veen, 2000)**

Stabilization of light- or oxygen-sensitive compounds
Stabilization of volatile compounds
Alteration of chemical reactivity
Improvement of solubility
Improvement of smell and taste
Modification of liquid compounds to powders

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1.5.2 Applications of cyclodextrins

The largest CD consumers are food and cosmetic industries. CDs are used to: solubilize and stabilize flavor/color/sensitive components; stabilize emulsions; reduce or eliminate the bad smells of certain components; reduce the loss of active components through volatilization, rapid oxidation, destruction by light, or improve the absorption of active components onto the skin. In household and toiletry products, the deodorizing capacity of CDs is utilized.

Less than 10 % of all produced CDs and CD derivatives are consumed by the pharmaindustry. Though minute amounts of cyclodextrins are used in drug formulations, the field of application has been most thoroughly studied. Cyclodextrins are used to increase stability, solubility, and bioavailability of drugs. The controlled released property is very useful both in drug and cosmetic fields.

In textile industry, CDs have various possibilities, but the blockbuster will be the chemical binding of CDs onto the surface of natural and synthetic fibers. The immobilized CDs can bind volatile molecules like the unpleasant components of sweat or cigarette smoke. When previously charged, the slow release of fragrances, insect repellents, or even drugs (for transdermal delivery) will be possible by wearing bonded CD-containing garments.

In chemical industry, the number of examples for application of CDs rapidly increases. For example, for conservation of wood products, water-insoluble fungicides have to be impregnated into the wood structures (door and window frames, etc.) Earlier, this was possible only by dissolving these water-insoluble fungicides in organic solvents, now it is possible to use simply aqueous cyclodextrin solutions for this purpose. To reduce the high viscosity of polyurethane thickening agent containing emulsion-type coatings (to facilitate the spraying), CDs are very appropriate, and by adding a small amount of very stable complex-forming detergents just before the spraying, the high viscosity can be restored within minutes. CDs can be used in hydrocarbon-polluted soil remediation: the CD mobilizes the insoluble polyaromatic hydrocarbons, bringing them into the aqueous phase, where the soil microorganisms can rapidly metabolize them. CDs, incorporated into packaging plastic films, strongly reduce the loss of the aroma substances by pervaporation. Or incorporating CD-complexed fungicides into the packaging films can elongate shelf-life of food

products. CDs have wide fields of utilization in sensors, in diagnostic kits, and in analytical chemistry, particularly in the chromatographic techniques (Szejtli, 2004).

Recently, cyclodextrins have been reported to help improving catalytic efficiency of certain enzymes in organic solvents (Santose et al., 1999; Hasegawa et al., 2003). Sometimes enzymes have to catalyze reactions in organic solvent such as in case of substrate which does not dissolve in aqueous solution or reaction which prefers no water system (Klibanov, 2001). In 1999, Griebenow and colleagues reported that activities in organic solvent of subtilisin Carlsberg were improved by co-lyophilization with methyl-β-cyclodextrin. Enzyme activity in transesterification reaction was 164 folds increased. By investigation using Fourier transform infrared (FTIR) spectroscopy, it is well established that lyophilization caused significant structural changes in subtilisin. Additive methyl-β-cyclodextrin causes subtilisin structural mobility ("flexibility"), and the more flexible the enzyme is, the greater the probability that the enzyme achieves a more active and enantioselective conformation. In another study, α-chymotrypsin was co-lyophilized with 2,3,6-tri-*O*-methyl β-cyclodextrin, the activity was 40 folds higher than free α-chymotrypsin for transesterification in acetonitrile (97:3) (v/v). Furthermore, α-chymotrypsin which was co-lyophilized with hydroxypropylated β- or γ- cyclodextrins retained more than 98% of its initial activity after 6 hr incubation in 97% acetonitrile. They suggested that association of α-chymotrypsin with CD may decrease the aggregation of α-chymotrypsin surface molecules, and this may be a consequence of stabilization of native structure of enzyme by hydrogen bonding with CD (Ooe et al.,1999). Moreover, in 2003, Mine and colleagues used cyclodextrins for enhanced enzyme activity and enantioselectivity of lipases in organic solvent. Lipase of *Pseudomonas cepacia* co-lyophilized with permethylate β-CD increased initial rate of transesterification by up to 17 folds and enantioselectivity could be doubled.

1.5.3 β-cyclodextrin safety status

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) considers β-CD as approved encapsulating agent for food additives, flavoring and vitamins; thickening agent with an ADI (acceptable dairy intake) of 0-5 mg/kg body weight (bw), based on the no-observed-effect level (NOEL) of 1.25% in the diet (equal to 470 mg/kg bw/day) in the one year study in dogs and a safety factor of 100

Table 6 Regulatory status of the natural cyclodextrins (January 2004) (Loftsson, 2005).

	Food approval			**Pharmacopoeia Monographs**		
	US	**Europe**	**Japan**	**USP/NF***	**Ph.Eur.****	**JP*****
α-CD	In preparation	Planned	Yes	No	Yes	Yes
β-CD	GRAS	Food additive	Yes	Yes	Yes	Yes
γ-CD	GRAS	Pending	Yes	No	In progress	Yes

* **United States Pharmacopoeia – National Formulary**

** **European Pharmacopoeia**

*** **Japanese Pharmacopoeia**

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(JECFA, 2005). Recently, β-CD has been recognized as GRAS (Generally Considered As Safe) for use as a flavor modifier in the food categories at use levels specified in the GRAS application submitted to the United States Food and Drug Administration (FDA) (Food Navigator, 2001). Table 6 shows summary of regulatory status of the natural cyclodextrins in different parts of the world (Loftsson, 2005).

1.6 β-galactosidase and cyclodextrin

As mentioned in 1.5.2, cyclodextrins have been reported to help improving catalytic efficiency of proteases (Griebenow et al., 1999; Ooe et al., 1999; Santose et al., 1999; Hasegawa et al., 2003) and lipases (Mine et al., 2003) in organic solvents. However, little is known about the applicability of cyclodextrins to glycosidases. Due to the widely accepted potential use of non-digested or functional oligosaccharides, a successful synthesis system is required. It is our interest to explore the possibility of using cyclodextrins for the improvement of galacto-oligosaccharide synthesis by β-galactosidase in organic solvent. In the present study, co-lyophilization of β-galactosidase with cyclodextrins from an aqueous solution will be performed. Then the co-lyophilizate will be used for oligosaccharide synthesis in appropriate organic solvent system and compared with free enzyme. Furthermore, optimization of oligosaccharide synthesis for high product yield will also be performed.

The objective of this study

1. To select appropriate organic solvent for synthetic reaction of β-galactosidase.
2. To study effect of cyclodextrin and its derivatives on oligosaccharide synthesis of β-galactosidase in organic solvent condition.
3. To optimize condition of oligosaccharide synthesis for β-galactosidase/cyclodextrin complex.
4. To examine the effect of cyclodextrin on the structure of β-galactosidase.

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CHAPTER II
MATERIALS AND METHODS

2.1 Equipments

Autopipette: Nichipet EX, Nichiryo, Japan
Balance: AB204-S, Mettler Toledo, Switzerland
Balance: PB303-S, Mettler Toledo, Switzerland
Electrophoresis unit: Model Mini-protein II Cell, BIO-RAD, USA
Electrospray Ionization-Time of Flight Mass Spectrometry (ESI-TOF MS): micrOTOF, Bruker, Germany
Fourier Transform Infrared (FT-IR) Spectroscopy: Perkin Elmer (Spectrum One), Perkin Elmer, UK
Fourier Transform Nuclear Magnetic Resonance (FT-NMR): INOVA-500, Varian, USA
High Performance Liquid Chromatography: Shimadzu LC-3A, Shimadzu, Japan
Imaging Densitometer: GS-670, BIO-RAD, USA
Luminescence spectrophotometer: Perkin Elmer LS 55, Perkin Elmer, UK
Lyophilizer: LYPH·LOCK, LABCONCO, USA
pH meter: pH900, Precisa, Switzerland
PLC Plates: Silica gel 60 F_{254} 1mm, Merck, Germany
Syringe: Holder 13 mm SST Swinney Syringe, Millipore, USA
TLC Plates: Silica gel 60, Merck, Germany
UV-VIS Spectrophotometer: DU650 Spectrophotometer, Beckman, USA
VIS Spectrophotometer: 6400 Spectrophotometer Jenway, LABQUIP, England
Vortex: Model K-550-GE, Scientific Industries, USA
Water Bath: Memmert, Germany

2.2 Chemicals

Acetic acid: BDH Chemical Ltd., England
Acrylamide: Merck, USA
Acetonitrile (HPLC grade): LAB-SCAN Analytical Science, Ireland
Ammonium persulfate: Pharmacia fine chemicals, Sweden

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Bromophenol blue: Merck, USA

1,4-Butanediol: Riedel-de Haën, Germany

Citric acid: Sigma, USA

Coomassie brilliant blue R-250: Sigma, USA

β-Cyclodextrin: Nihon Shokuhin Kako Company Ltd., Japan

Ethyl acetate: Scharlau, Spain

Triethyl phosphate: Fluka, Germany

D(+)-Galactose: BDH Chemical Ltd., England

β-Galactosidase from *Aspergillus oryzae*: Sigma, USA

D-Glucose anhydrous: APS Ajax Finechem, Australia

Glycerol: APS Ajax Finechem, Australia

Glycine: Sigma, USA

Hydrochloric acid: LAB-SCAN Analytical Science, Ireland

Hydroxypropyl-β-Cyclodextrin: Ensuiko sugar refining co.,Ltd., Japan

Lactose: APS Ajax Finechem, Australia

2-Mercaptoethanol: Scharlau, Spain

Methanol: Scharlau, Spain

2-Methoxyethyl acetate: Fluka, Germany

N,N′-methylene-bis-acrylamide: Sigma, USA

Methyl-β-Cyclodextrin: Ensuiko sugar refining co.,Ltd., Japan

O-Nitrophenol: Merck, Germany

O-Nitrophenyl-β-D-Galactopyranoside: Sigma, USA

1,5-Pentanediol: Fluka, Germany

n-Propyl Alcohol: Carlo Erba reagents, Germany

Sodium acetate trihydrate: BDH Chemical Ltd., England

Sodium Carbonate anhydrous: Riedel-de Haën, Germany

Tri-Sodium Citrate dehydrate: Carlo Erba reagents, Germany

Sodium dodecyl sulfate: Sigma, USA

Di-Sodium hydrogen phosphate dehydrate: Fluka, Germany

Sodium phosphate monohydrate: Carlo Erba reagent, Germany

Standard molecular weight marker protein: Amersham, USA

Sulphuric acid: BDH Chemical Ltd., England

TEMED (*N,N,N′,N′*- tetramethylene-ethylenediamine): Fluka, Germany

Tris(hydroxymethyl)-aminomethane: UBS, USA

2.3 Protein determination

Protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as the standard.

2.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The denaturing gel was performed according to Bollag and Edelstein (1991). The gel was carried out with 0.1% (w/v) SDS in 7.5% (w/v) separating and 5.0% (w/v) stacking gel and Tris-glycine buffer pH 8.0 containing 0.1% SDS was used as electrode buffer (see Appendix 1). Sample to be analyzed was treated with sample buffer and boiled for 5 minutes prior to application to the gel. The electrophoresis was performed at constant current of 20 mA per slab, at room temperature on a Mini-Gel electrophoresis unit from cathode towards anode.

2.4.1 Coomassie blue staining

Gel was stained with 0.1% (w/v) of Coomassie brilliant blue R-250 in 45% (v/v) methanol and 10% (v/v) acetic acid for at least 2 hours. The slab gel was destained with a solution of 10% methanol and 10% acetic acid for 1-2 hours, followed by several changes of destaining solution unit gel background was clear.

2.5 Enzyme assay

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β-Galactosidase activity was determined by assays of hydrolysis activity and oligosaccharide synthetic activity.

2.5.1 Hydrolysis activity

Hydrolysis activity of β-galactosidase was measured by slight modification method of Switzer and Garrity (1999) and Gaur et al. (2006).

Enzyme (0.050 mg, in soluble or lyophilized form) was incubated with 3 ml of substrate (1.25 μmol of *O*-nitrophenyl-β-D-galactopyranoside (ONPG) in 50 mM citrate buffer pH 4.5 or in mixture of organic solvents*) at 30°C for 5 minutes.

The reaction was stopped with 2 ml of 1 M sodium carbonate and the absorbance at 420 nm was measured. For control, enzyme was not added.

One unit of enzyme was defined as the amount of enzyme which produces 1 μmol of *O*-nitrophenol per minute under the described condition.

* Organic solvents used were: 1,4-butanediol, 1,5-pentanediol, methoxyethyl acetate, triethyl phosphate, and acetonitrile. They were mixed at various ratios with 50 mM citrate buffer and used as co-solvents.

2.5.2 Oligosaccharide synthesis activity

Oligosaccharide synthesis was determined by slight modification method of Iwasaki et al. (1994).

The reaction mixture, containing 0.025 mg of enzyme (in soluble or lyophilized form) and 1.5 ml of substrate (0.15 M lactose in 50 mM citrate buffer pH 4.5 or in mixture of organic solvents*) was incubated at 40°C for 48 hours. The reaction was stopped by boiling for 10 minutes. Measuring the synthesized oligosaccharides was by thin layer chromatography (TLC) or high performance liquid chromatography (HPLC) as described in section 2.6.

* The same as in 2.5.1.

2.6 Oligosaccharide determination

Oligosaccharide was measured by TLC or HPLC. Percent product yield was calculated from peak area and expressed as:

$$\text{Yield (\%)} = \frac{\text{Amount of synthesized oligosaccharide}}{\text{Amount of lactose substrate}} \times 100$$

2.6.1 Thin layer chromatography (TLC)

The determination of oligosaccharides by TLC was performed as described by Dawson et al. (1986). The TLC system was n-propanol : ethyl acetate : water (7:1:2) by volume. Detection of components on a thin layer chromatogram can readily be achieved using a non-selective charring technique by heating the

chromatogram at 110°C for 15 minutes after spraying it with a reagent prepared from concentrated sulphuric acid : ethanol (1:9) (Kennedy and Pagliuca, 1994). Quantitative analysis of oligosaccharides on TLC plate was by BIO-RAD Imaging Densitometer GS-670. Product yield was calculated from peak area.

2.6.2 High performance liquid chromatography (HPLC)

The analysis of oligosaccharides by HPLC was slightly modified from the method of Maitin et al. (2004). The HPLC system was a Shimadzu LC-3A equipped with Lichrocart-NH_2 column (0.46 x 25 cm) and using Shimadzu RID-3A refractometer as detector. The reaction mixture of oligosaccharide synthetic reaction was filtered through a 13 mm Nylon 0.45 μm disc filter before injection and eluted with acetonitrile : water (75:25, v/v) using a flow rate of 1 ml/min. The oligosaccharide peaks were identified by comparing the retention times with those of standard glucose, galactose and lactose (10 μg/ml). Product yield was calculated from peak area.

2.7 Co-lyophilization of β-galactosidase with cyclodextrin or its derivatives

For control condition, enzyme (0.050 mg for hydrolysis reaction and 0.025 mg for synthesis reaction) was dissolved in 50 mM citrate buffer pH 4.5 and lyophilized for 24 hours. Co-lyophilization with cyclodextrin or its derivatives was performed as in control, except that the excipient was added prior to lyophilization. After lyophilization, the enzyme preparation was suspended in citrate buffer for activity determination as mentioned in 2.5.1 or 2.5.2 or stored at -20°C for further use.

2.7.1 Effect of co-lyophilization of β-galactosidase with cyclodextrin or its derivatives on oligosaccharide synthesis

β-galactosidase was lyophilized with cyclodextrin or its derivatives in citrate buffer pH 4.5 as described in section 2.7. The cyclodextrins tested were as follows: β-cyclodextrin, hydroxypropyl-β-cyclodextrin and methyl-β-cyclodextrin. In addition, short-chain linear oligosaccharides, G_5 and G_7, were also used. Then,

lyophilized β-galactosidase was subjected to oligosaccharide synthesis as described in section 2.5.2

2.7.2 Effect of ration of β-galactosidase to cyclodextrin co-lyophilized on oligosaccharide synthesis

β-galactosidase was lyophilized with β-cyclodextrin in citrate buffer pH 4.5 as described in section 2.7. The ratio of enzyme : cyclodextrin by weight used were as follows : 1:5, 1:10, 1:20, 1:40, 1:60, 1:80 and 1:100. Then, lyophilized β-galactosidase was subjected to oligosaccharide synthesis as described in section 2.5.2.

2.8 Optimization of oligosaccharide synthesis

2.8.1 Effect of lactose

The co-lyophilized enzyme (0.025 mg, 0.125 Units) was incubated with various concentrations of lactose in 50 mM citrate buffer pH 4.5 or in a mixture of selected organic solvent at 40°C for 48 hours. The concentrations of lactose used were as follows: 0.3 M, 0.25 M, 0.20 M, 0.15 M, 0.10 M, 0.05 M and 0 M. The reaction was stopped by boiling for 10 minutes, and then analyzed as described in section 2.6.

2.8.2 Effect of enzyme

Various amounts of enzyme were incubated with optimal concentration of lactose in 50 mM citrate buffer pH 4.5 or in a mixture of selected organic solvent at 40°C for 48 hours. The amounts of enzyme used were as follows: 0 mg, 0.00625 mg, 0.0125 mg, 0.025 mg, 0.0625 mg, 0.125 mg and 0.250 mg (0-1.25 Units). The reaction was stopped by boiling for 10 minutes, and then analyzed as described in section 2.6.

2.8.3 Effect of pH

Optimal amounts of enzyme and lactose were incubated in 50 mM buffer solution or in a mixture of selected solvent in the pH range of 3.0-8.0 at 40°C for 48 hours (amount of enzyme and concentration of lactose were obtained from results of section 2.8.1 and 2.8.2). The buffers used were as follow: citrate buffer pH 3-5, acetate buffer pH 5-6 and phosphate buffer pH 6-8. The reaction was stopped by boiling for 10 minutes, and then analyzed as described in section 2.6.

2.8.4 Effect of temperature

Optimal amounts of enzyme and lactose were incubated in 50 mM buffer solution or in a mixture of selected solvent at optimal pH (amount of enzyme, concentration of lactose and pH were obtained from results of section 2.8.1-2.8.3) at various temperatures for 48 hours. The temperatures used were as follow: 30, 40, 50, 60 and 70°C. The reaction was stopped by boiling for 10 minutes, and then analyzed as described in section 2.6.

2.8.5 Effect of reaction time

Optimal amount of enzyme and lactose were incubated in 50 mM buffer solution or in a mixture of selected solvent at optimal pH and temperature (amount of enzyme, concentration of lactose, pH and temperature were obtained from results of section 2.8.1-2.8.4) at reaction time of 0-96 hours. The reaction was stopped by boiling for 10 minutes, then analyzed as described in section 2.6.

2.9 Measurement of fluorescence spectrum of β-galactosidase/cyclodextrin complex

Fluorescence spectrum was measured by the method of Yu et al. (2001). Fluorescence spectrum of lyophilized β-galactosidase/cyclodextrin complex was determined in comparison with lyophilized β-galactosidase in the absence of cyclodextrin and non-lyophilized (soluble) β-galactosidase. β-galactosidase was co-lyophilized with cyclodextrin or derivatives in citrate buffer pH 4.5 as described in

section 2.7. The cyclodextrin and derivatives used were as follows: β-cyclodextrin, hydroxypropyl-β-cyclodextrin and methyl-β-cyclodextrin. Then, the fluorescence spectrum were determined by Perkin Elmer Luminescence spectrophotometer LS 55. Excitation wavelength was at 280 nm while emission was performed in the range of 290-450 nm.

2.10 Fourier transform infrared (FT-IR) spectroscopy

FT-IR spectroscopy was measured by the method of Griebenow et al. (1999). FT-IR spectroscopy of lyophilized β-galactosidase/β-cyclodextrin complex was determined in comparison with lyophilized β-galactosidase in the absence of β-cyclodextrin and non-lyophilized β-galactosidase. β-galactosidase was co-lyophilized with β-cyclodextrin in citrate buffer pH 4.5 as described in section 2.7. Then, the FT-IR spectrum were determined by Perkin Elmer Spectroscopy (Spectrum One) at the Scientific and Technological Research Equipment Center of Chulalongkorn University.

2.11 Preparative layer plate (PLC)

The preparation of oligosaccharide products by PLC was performed using the same condition as in the analytical TLC (section 2.6.1). The oligosaccharides were extracted by distilled water after the spots have been scraped from the layer.

2.12 Mass spectrometry (MS)

Electrospray Ionization-Time of Flight Mass Spectrometry (ESI-TOF MS) profile was recorded on a micrOTOF at the Service Unit of the National Center for Genetic Engineering and Biotechnology. The mixture of methanol : H_2O was used as solvent.

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2.13 Nuclear magnetic resonance (NMR)

^{1}H and ^{13}C NMR spectrum were determined using a Varian INOVA-500 (FT-NMR) spectrometer at the Scientific and Technological Research Equipment Center of Chulalongkorn University. The operation was at 500 MHz for protons and 125 MHz for carbons. The chemical shifts were expressed in ppm downfield from the signal of tetramethylsilane (TMS), which was used as internal standard.

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CHAPTER III

RESULTS

3.1 Purity of β-galactosidase

β-galactosidase of *Aspergillus oryzae* is a commercial product of Sigma, USA. The purity of β-galactosidase enzyme was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described in section 2.4. Protein staining revealed that the enzyme was purified since one protein band was observed from Coomassie brilliant blue staining of the gel (Figure 6, lane 2 and 3). Hydrolysis activity was measured as described, and 5 U/mg specific activity was obtained.

3.2 Selection of most appropriate organic solvent

3.2.1 Determination of hydrolysis activity of β-galactosidase

The effect of organic solvents on hydrolysis activity of β-galactosidase was measured as described in section 2.5.1. Organic solvents used are water miscible: 1,4-butanediol, 1,5-pentanediol, methoxyethyl acetate, triethyl phosphate and acetonitrile. They were prepared as co-solvents by mixing with various proportions of 50 mM citrate buffer, pH 4.5. However, in some organic solvents e.g. triethyl phosphate, methoxyethyl acetate and acetonitrile, the high ratio of organic solvent made reaction mixture change to emulsion, so hydrolysis activity could not be determined by measuring absorbance at 420 nm. The maximum ratio of each solvent which could be prepared as complete miscible solvent in buffer was: 1,4-butanediol (80:20), 1,5-pentanediol (60:40), methoxyethyl acetate (50:50), triethyl phosphate (20:80) and acetonitrile (40:60) (v/v). The hydrolysis activity of β-galactosidase in these solvents was determined in relative to that in 50 mM citrate buffer, pH 4.5 which was set as 100% activity (Figure 7). The ratio of organic solvent had a significant effect on hydrolysis activity of β-galactosidase. When the ratio of organic solvent was increased, hydrolysis activity was decreased. And when hydrolysis

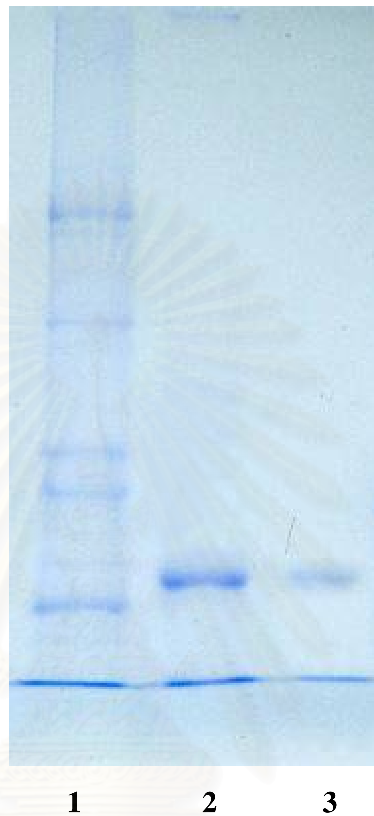

Figure 6 **SDS-PAGE of β-galactosidase**

Lane 1: Protein molecular weight markers (Myosin, 220 kDa ; α-2-Macroglobulin, 170 kDa ; β-galactosidase, 116 kDa ; Transferrin, 76 kDa ; Glutamate dehydrogenase, 53 kDa)

Lane 2: β-galactosidase (20 μg)

Lane 3: β-galactosidase (10 μg)

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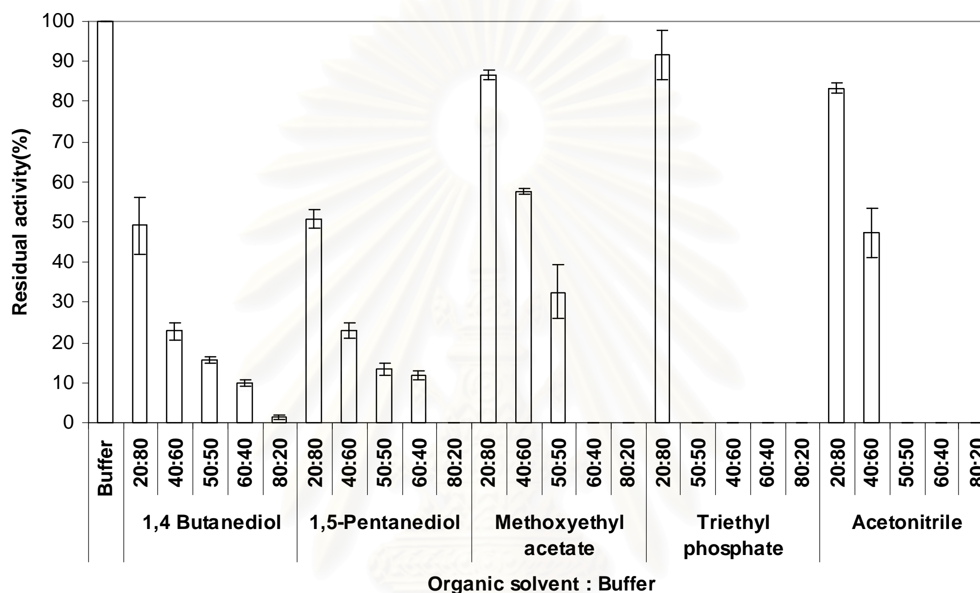

Figure 7 **Hydrolysis activity of β-galactosidase in organic solvent mixtures.** Residual activity (%) was relative to the hydrolysis activity of β-galactosidase in 50 mM citrate buffer solution, pH 4.5. All values were average from three separate experiments.

Note: 1,5-pentanediol (80:20), methoxyethyl acetate (60:40, 80:20), triethly phosphate (50:50, 40:60, 60:40, 80:20) and acetonitrile (50:50, 60:40, 80:20) co-solvents turned reaction mixture into emulsion, so activity could not be measured.

activities in different solvents at 20:80 solvent : buffer ratio were compared, 84-92% activity in relative to that in aqueous buffer solution was obtained with triethyl phosphate, methoxyethyl acetate and acetonitrile, while only about 50% activity was left in 1,4-butanediol and 1,5-pentanediol. At higher solvent ratio (40:60), triethyl phosphate co-solvent became emulsion while activity in methoxyethyl acetate was higher than in acetonitrile. These results suggest that methoxyethyl acetate, triethyl phosphate and acetonitrile were good solvents for hydrolysis activity of β-galactosidase.

3.2.2 Determination of oligosaccharide synthetic activity of β-galactosidase

The effect of organic solvents on oligosaccharides synthesis by β-galactosidase was determined as described in section 2.5.2. The organic solvents and the ratio used were from the result in section 3.2.1, the maximum ratio of each solvent was used except for the two alcohols, of which the 50:50 ratio was used : 1,4-butanediol (50:50), 1,5-pentanediol (50:50), methoxyethyl acetate (50:50), triethyl phosphate (20:80) and acetonitrile (40:60) (v/v). Measuring the synthesized oligosaccharides was by TLC method as described in section 2.6.1. The result (Figure 8) shows that only in methoxyethyl acetate (50:50) and triethyl phosphate (20:80) (lane e and g, respectively), the enzyme could synthesize oligosaccharide product while in 1,4-butanediol (50:50), 1,5-pentanediol (50:50) and acetonitrile (40:60) (v/v), no product was observed. The product, named Oligo-I, had the Rf of 0.19.

Methoxyethyl acetate (MEA) and triethyl phosphate (TEP) were chosen for varying solvent ratios and determined for oligosaccharide synthesis. In the synthetic assay, 50%TEP could be used without emulsion problem as occurred in the hydrolysis activity assay. Two ratios, 50:50 and 20:80 (organic solvent:buffer) (v/v), were used. The oligosaccharide product was detected by both TLC (Figure 9) and HPLC. Oligo-I was observed in all solvents on TLC plates. The order of spot intensity was, 50%TEP > 20%TEP > 50%MEA > 20%MEA. For separation by HPLC, the chromatograms of reaction mixtures in MEA and TEP gave 3 peaks (Figure 10), the retention times of galactose/glucose, lactose, and oligosaccharide product (Oligo-I) were 4.7, 6.1 and 8.0 minutes, respectively. The peak at 3.1 minutes was the solvent peak. Product yield (%) was then calculated from peak area. The result (Figure 11)

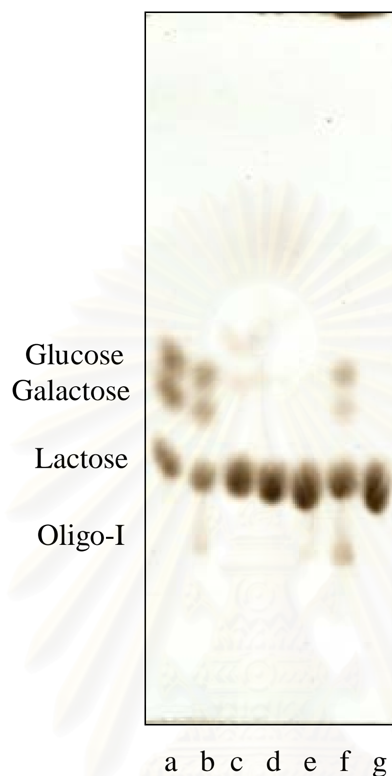

Figure 8 **TLC chromatogram of synthetic reaction products of β-galactosidase in co- solvents.**

a) Standard glucose, galactose, and lactose (20 μg each)

Synthetic reaction products in, b) buffer solution;

c) 1,4-butanediol (50:50); d) 1,5-pentanediol (50:50);

e) methoxyethyl acetate (50:50); f) triethyl phosphate (20:80),

g) acetonitrile (40:60)

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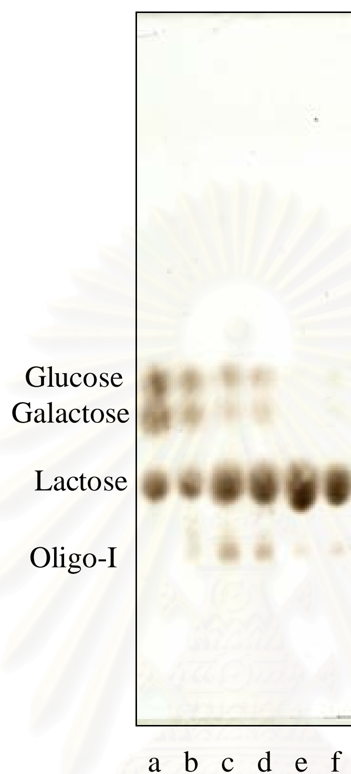

Figure 9 **TLC chromatogram of synthetic reaction products of β-galactosidase in triethyl phosphate and methoxyethyl acetate co-solvent.**

a) Standard glucose, galactose, and lactose (20 μg each)

Synthetic reaction products in, b) buffer solution;

c) triethyl phosphate (50:50); d) triethyl phosphate (20:80);

e) methoxyethyl acetate (50:50), f) methoxyethyl acetate (20:80)

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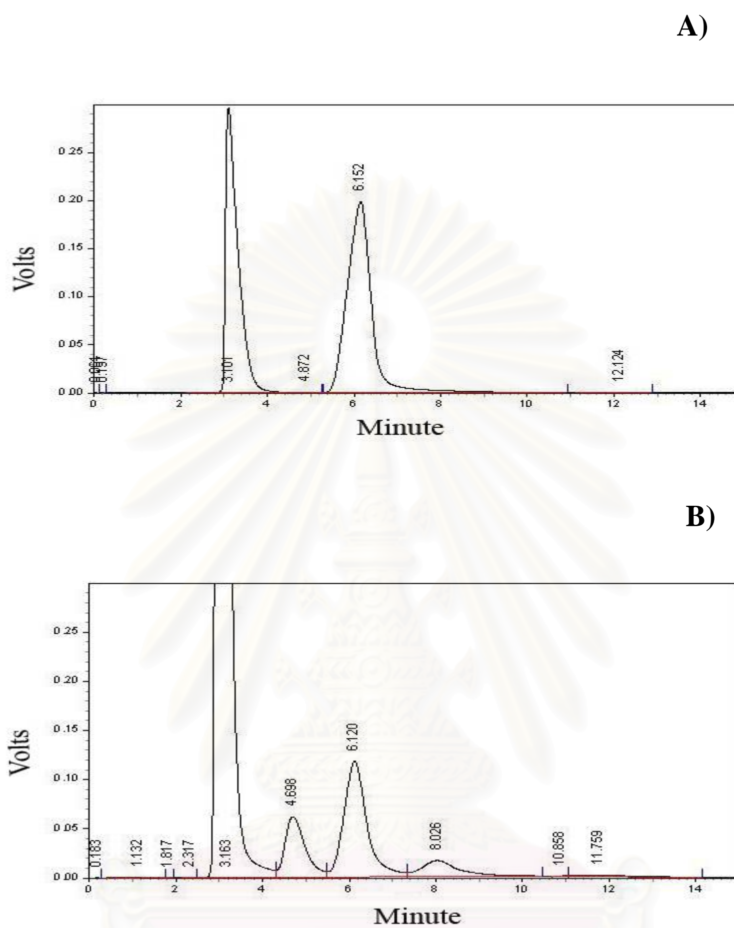

Figure 10 **HPLC chromatograms of synthetic reaction products of β-galactosidase in 50%TEP co-solvent at incubation time of A) 0 hour ; B) 48 hours.**

Retention time	4.7 minutes	galactose/glucose peak
Retention time	6.1 minutes	lactose peak
Retention time	8.0 minutes	Oligo-I peak

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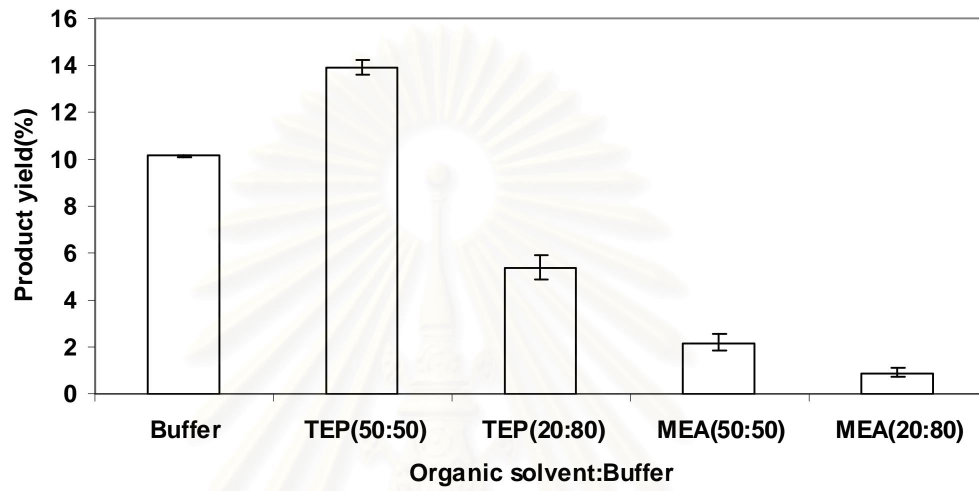

Figure 11 Oligosaccharide synthesis by β-galactosidase in different solvents.

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shows that the highest amount of synthesized Oligo-I was detected in 50%TEP co-solvent. Product yield was 13.5% which was 1.4 and 6.8 times higher than the yield in buffer and 50%MEA, respectively.

3.2.3 Determination of hydrolysis activity of lyophilized β-galactosidase

The purpose of this experiment was to observe the effect of lyophilization on the hydrolysis activity of β-galactosidase. This experiment was performed by lyophilizing the enzyme from aqueous solution of 50 mM sodium acetate buffer (pH 4.5) as described in section 2.7. Then, the effect of organic solvent on hydrolysis activity of lyophilized β-galactosidase as compared to the activity of enzyme in solution was measured as described in section 2.5.1. Organic solvents used were the same as in section 3.2.1. They were: 1,4-butanediol (50:50), 1,5-pentanediol (50:50), methoxyethyl acetate (50:50), triethyl phosphate (20:80) and acetonitrile (40:60) (v/v). The result (Figure 12) shows that the hydrolysis activity in buffer of the lyophilized enzyme was 33% decreased when compared with hydrolysis activity of non-lyophilized enzyme and the average value of activity decrease upon lyophilization from all assay systems was approximately 45%. When hydrolysis activity was determined in different solvent systems, the highest activity was observed with 20% TEP co-solvent, both for the non-lyophilized and lyophilized enzyme forms.

3.2.4 Determination of oligosaccharide synthetic activity of lyophilized β-galactosidase

From the results of section 3.2.1-3.2.3, 50% TEP co-solvent seemed to be the most appropriate solvent for β-galactosidase synthesis activity. The purpose of this experiment was to investigate the effect of lyophilization of the enzyme on the synthetic activity in buffer and 50% TEP co-solvent. The result (Figure 13) shows that the highest amount of the synthesized oligosaccharide was observed in 50% TEP. It should be noted that oligosaccharide synthesis was not affected by lyophilization. Product yield was 18.5% for both the lyophilized and lyophilized enzyme in 50%TEP

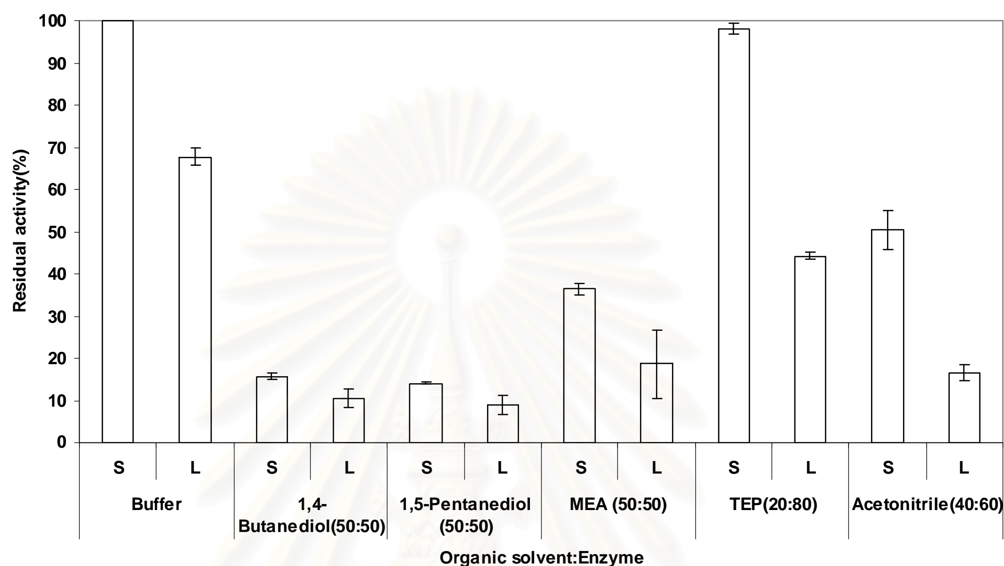

Figure 12 **Hydrolysis activity of lyophilized β-galactosidase in organic solvent mixtures.** Residual activity (%) was relative to the hydrolysis activity of non-lyophilized β-galactosidase in 50 mM citrate buffer solution, pH 4.5.

S	=	Soluble (non-lyophilized) enzyme
L	=	Lyophilized enzyme

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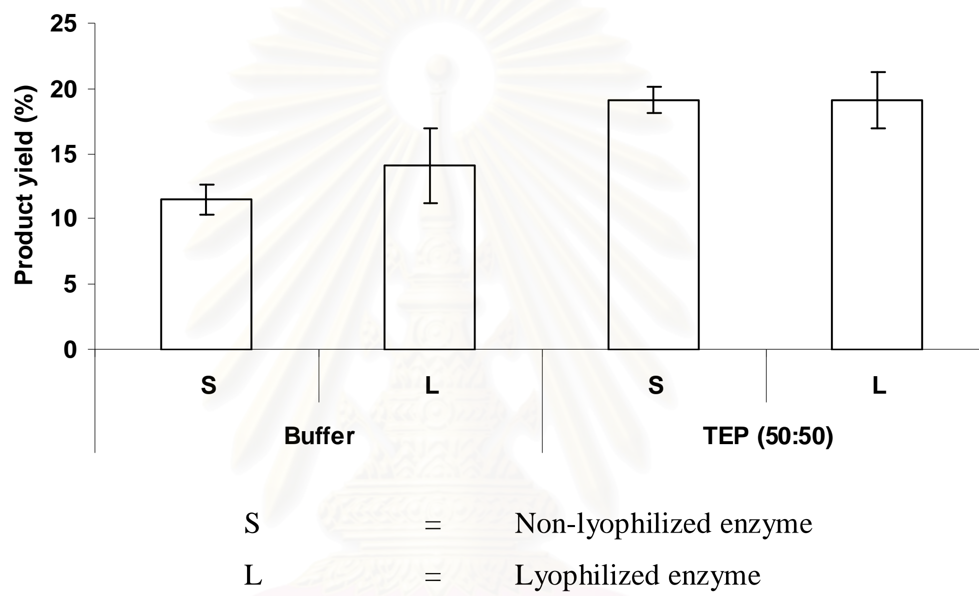

Figure 13 **Oligosaccharide synthesis by non-lyophilized and lyophilized β-galactosidase.**

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co-solvent. The increase was approximately 66.3% over the yield in buffer by non-lyophilized enzyme.

From the results in section 3.2.1-3.2.4, 50% TEP co-solvent was the best solvent system chosen for further experiments in the attempt to increase oligosaccharide synthetic ability of β-galactosidase.

3.3 Effect of cycledextrins on oligosaccharide synthesis by β-galactosidase in the presence of organic solvent

In the attempt to increase oligosaccharide synthetic ability of β-galactosidase, the enzyme was lyophilized in the presence of cyclodextrins as described in section 2.7. Then, lyophilized enzyme was investigated for oligosaccharide synthesis in buffer solution and in 50%TEP co-solvent.

3.3.1 Effect of type of cyclodextrins

For control, β-galactosidase was lyophilized from aqueous solution of citrate buffer, pH 4.5. Co-lyophilization with cyclodextrins was performed as described in section 2.7. The cyclodextrins used were: β-cyclodextrin, hydroxypropyl-β-cyclodextrin and methyl-β-cyclodextrin. In addition, short-chain linear oligosaccharides: G_5 and G_7 were also tested. The ratio of enzyme : cyclodextrin by weight was 1:5, then, lyophilized β-galactosidase was used for oligosaccharide synthesis in 50%TEP co-solvent as compared with synthesis in buffer solution. The synthesized oligosaccharides were determined by TLC and HPLC, and product yields were calculated. The result from TLC (Figure 14) shows that Oligo-I was produced in all lanes, the amount synthesized in 50%TEP was significantly higher than in buffer. From HPLC analysis (Figure 15) the product yield of oligosaccharide from β-galactosidase/ β-cyclodextrin complex was the highest when synthesis was in 50%TEP co-solvent. Hydroxypropyl-β-cyclodextrin, methyl-β-cyclodextrin, G_5 and G_7 also increased synthesis activity of lyophilized enzyme to a similar extent. It should be noted that oligosaccharide synthesis in buffer solution yielded products approximately two times less than in 50%TEP co-solvent. And, cyclodextrins and linear oligosaccharides didn't show effect on synthesis in buffer as

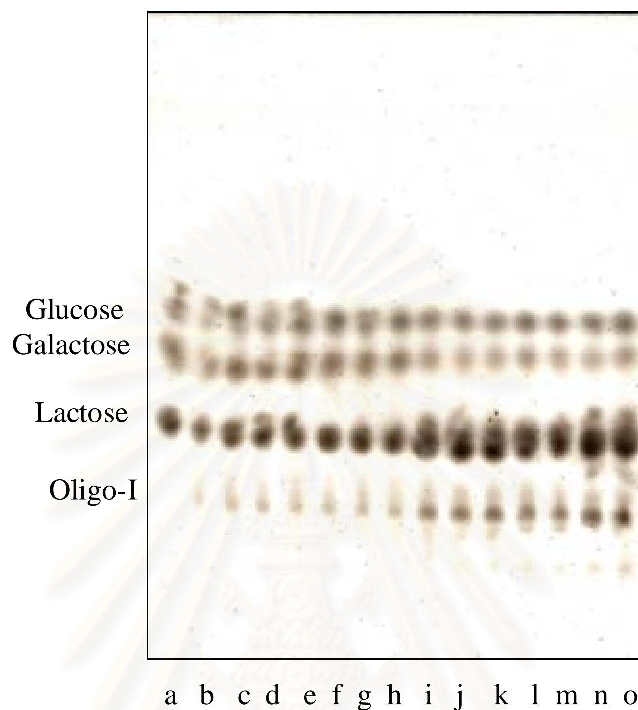

Figure 14 TLC chromatogram of synthetic reaction products of β-galactosidase lyophilized with cyclodextrins and linear oligosaccharides.

a) Standard glucose, galactose, and lactose (20 µg each)

Synthetic reaction products in buffer (b-h) and in 50%TEP (i-o).

Catalysis was by,

b and i) non-lyophilized enzyme;

c and j) lyophilized enzyme;

d and k) lyophilized enzyme with β-cyclodextrin;

e and l) lyophilized enzyme with hydroxypropyl-β-cyclodextrin ;

f and m) lyophilized enzyme with methyl-β-cyclodextrin ;

g and n) lyophilized enzyme with G_5 ;

h and o) lyophilized enzyme with G_7 ;

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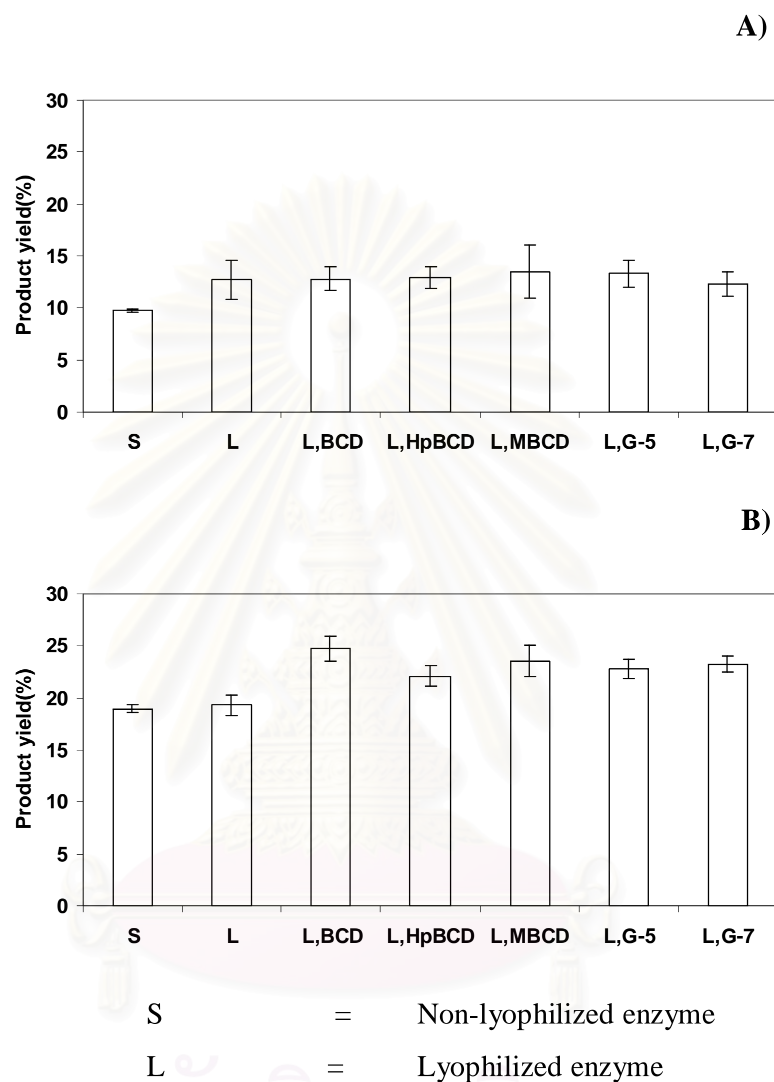

S = Non-lyophilized enzyme

L = Lyophilized enzyme

Figure 15 **Oligosaccharide synthesis by β-galactosidase co-lyophilized with cyclodextrins or linear oligosaccharides. Synthesis was in A) buffer; B) 50%TEP co-solvent.** The ratio of β-galactosidase to oligosaccharides was 1:5 w/w.

they did in TEP. According to the result, β-cyclodextrin was chosen as the best excipient added.

3.3.2 Effect of ratio of β-galactosidase to β-cyclodextrin

To determine suitable ratio of β-galactosidase to β-cyclodextrin, β-galactosidase was lyophilized with various ratios of enzyme to β-cyclodextrin from citrate buffer pH 4.5 as described. The ratio of enzyme : β-cyclodextrin by weight was varying from: 1:5, 1:10, 1:20, 1:40, 1:60, 1:80 and 1:100. Then, lyophilized β-galactosidase was used for oligosaccharide synthesis and product yield was determined as in section 3.3.1.

The results of TLC (Figure 16) and HPLC (Figure 17) show that product yield was the highest when used β-galactosidase : β-cyclodextrin co-lyophilizate at the ratios of 1:5 and 1:10. For higher ratio, synthesis was about the same level or even lower when compared to that of lyophilized β-galactosidase in the absence of β-cyclodextrin. According to the results, the appropriate ratio of enzyme : β-cyclodextrin was 1:5 by weight. This ratio was used for further experiments.

3.4 Optimization of oligosaccharide synthesis

The optimal condition for oligosaccharide synthesis using lyophilized enzyme was investigated. The lyophilized enzyme was prepared as β-galactosidase-β-cyclodextrin co-lyophilizate at the ratio of 1:5.

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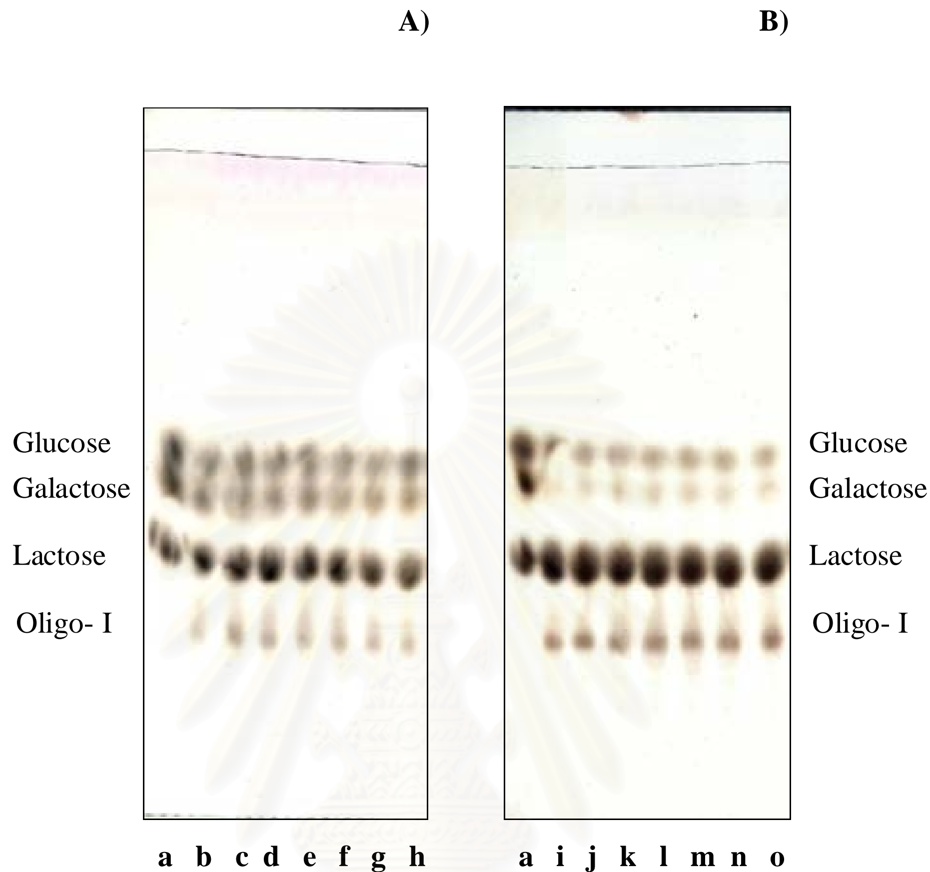

Figure 16 **TLC chromatogram of synthetic reaction products of β-galactosidase co-lyophilized with various ratios of β-cyclodextrin.**

Synthesis was in A) buffer ; B) 50%TEP co-solvent.

a) Standard glucose, galactose, and lactose (20 µg each)

Synthetic reaction products by,

b and i) non-lyophilized enzyme ;

c and j) lyophilized enzyme;

d and k) β-galactosidase:β-cyclodextrin ratio 1:5;

e and l) β-galactosidase:β-cyclodextrin ratio 1:10;

f and m) β-galactosidase:β-cyclodextrin ratio 1:20;

g and n) β-galactosidase:β-cyclodextrin ratio 1:40;

h and o) β-galactosidase:β-cyclodextrin ratio 1:80;

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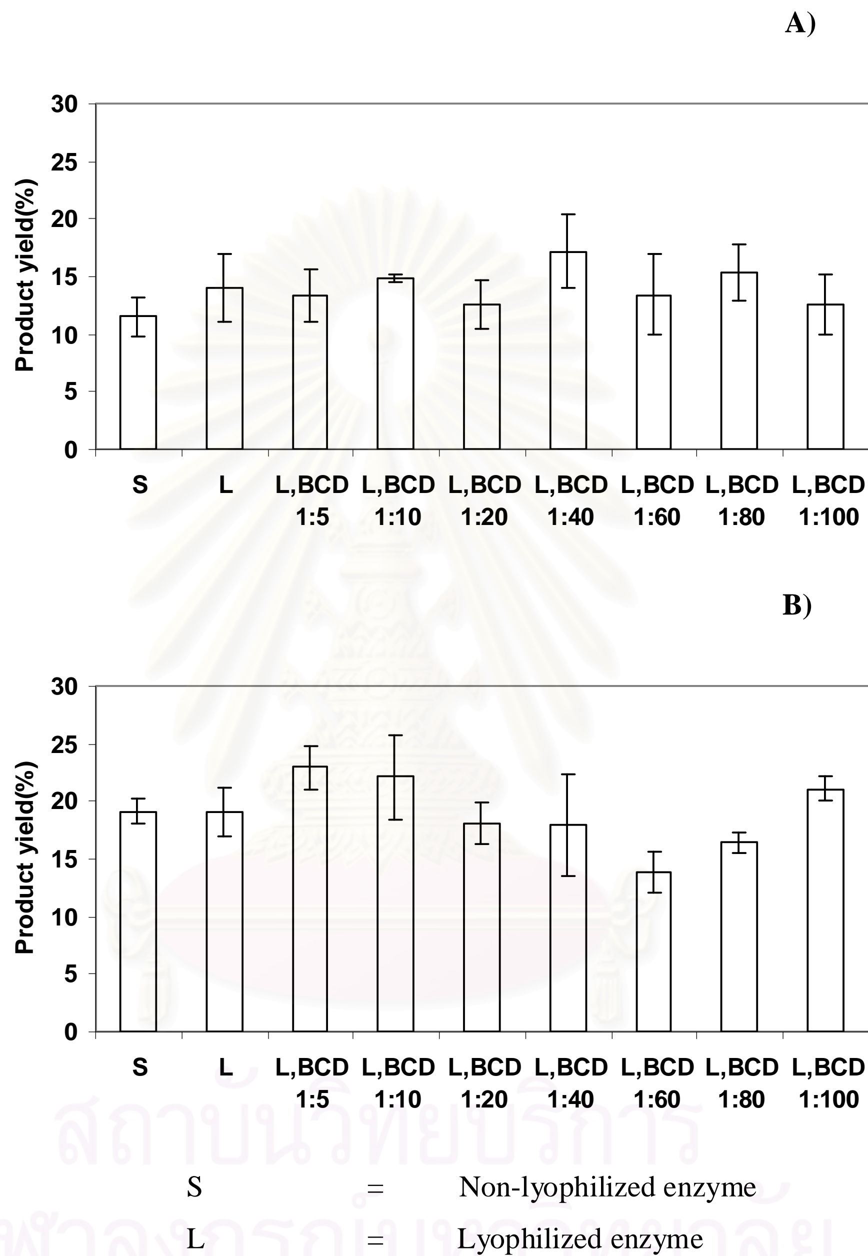

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Figure 17 **Oligosaccharide synthesis by various ratios of β-galactosidase-β-cyclodextrin co-lyophilizate.**

Synthesis was in A) buffer ; B) 50%TEP co-solvent

3.4.1 Effect of lactose concentration

The effect of substrate (lactose) concentration on oligosaccharide synthesis was determined as described in section 2.9.1. The concentration of lactose was varied from 0-0.3 M and the synthesis was performed using 0.025 mg (0.125 U, hydrolysis unit) β-galactosidase in 50%TEP co-solvent, pH 4.5 at 40°C for 48 hours. The synthesized oligosaccharide was detected by TLC as described in section 2.6.1.

TLC chromatogram (Figure 18) shows two synthesized oligosaccharides, the major one with the Rf of 0.19 (Oligo-I) and the minor (Oligo-II) at Rf 0.10. At 0.1-0.15 M lactose, only Oligo-I was observed, while at lower lactose concentration, the product was hardly seen, and Oligo-II was clearly seen when 0.2-0.3 M lactose was used. The density on chromatogram was analyzed by Imaging Densitometer and interpreted as percent product yield (Figure 19). The concentration of lactose which gave the maximum yield of oligosaccharide product (Oligo-I) was 0.3 M.

3.4.2 Effect of amount of enzyme

The effect of enzyme quantity on oligosaccharide synthesis was determined as described. The amount of enzyme was varied from 0-0.250 mg (0-1.25 U). The reactions were performed by incubating various amounts of enzyme with 0.3 M lactose in 50%TEP co-solvent, pH 4.5 at 40°C for 48 hours. The synthesized oligosaccharide was detected by TLC method.

TLC chromatogram (Figure 20) shows the synthesized oligosaccharides as one major spot at Rf 0.19 (Oligo-I) and one minor at Rf 0.10 (Oligo-II). For the lower amount of enzyme, only Oligo-I was seen, while Oligo-II was clearly seen when 0.025-0.0625 mg enzyme was used (lane e and d). When high amount of enzyme was used (lane b and c), in addition to Oligo-I and Oligo-II, a new spot emerging between the two. The density on chromatogram was analyzed by Imaging Densitometer and interpreted as percent product yield (Figure 21). It was found that the amount of enzyme which gave the maximum yield of oligosaccharide product (Oligo-I) was 0.025 mg.

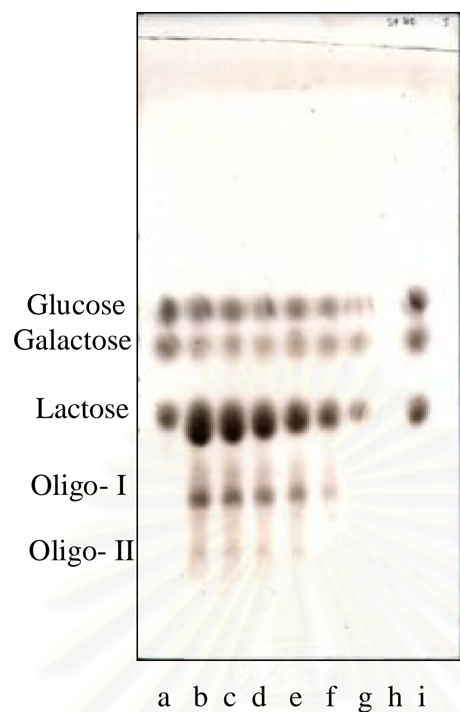

Figure 18 TLC Chromatogram of oligosaccharides synthesized in various concentrations of lactose.

a) and i) Standard glucose, galactose, and lactose (20 µg each)

Synthetic reaction products in lactose, b) 0.30 M; c) 0.25 M; d) 0.20 M; e) 0.15 M; f) 0.10 M; g) 0.05 M; h) 0 M

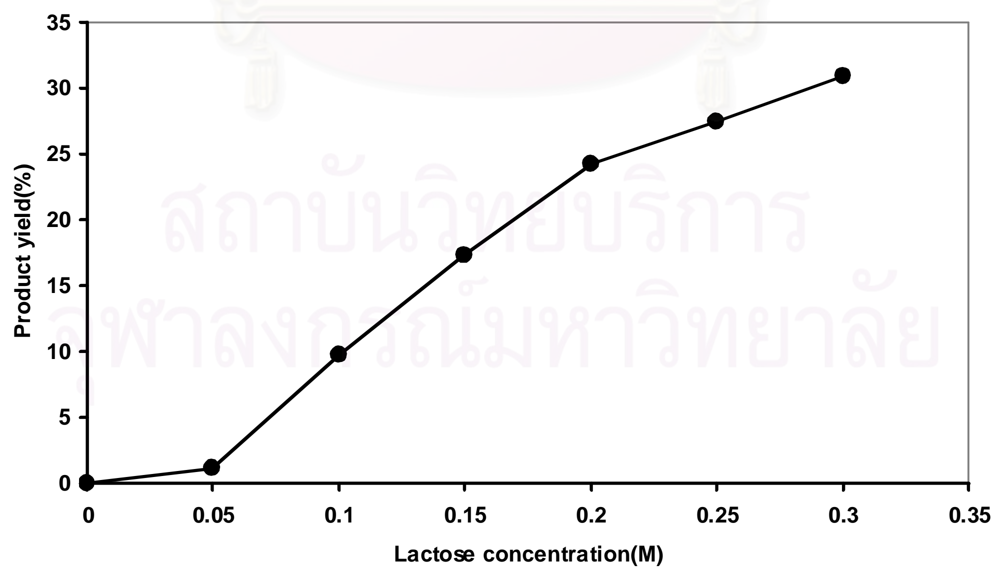

Figure 19 Effect of lactose concentration on oligosaccharide synthesis.

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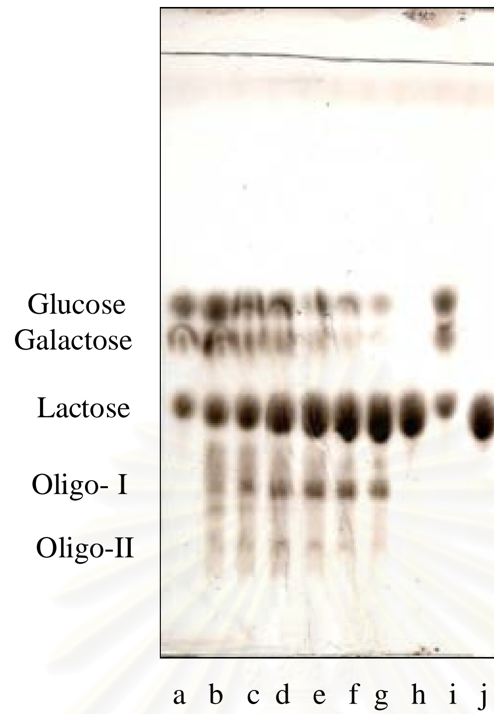

Figure 20 TLC chromatogram of oligosaccharides synthesized in various amounts of enzyme.

a) and i) Standard glucose, galactose, and lactose (20 µg each)

j) Synthetic reaction products at time 0 hour

Synthetic reaction products by enzyme, b) 0.250 mg (1.25 U); c) 0.125 mg (0.625 U); d) 0.0625 mg (0.3125 U); e) 0.025 mg (0.125U); f) 0.0125 mg (0.0625 U); g) 0.00625 mg (0.03125 U); h) 0 mg

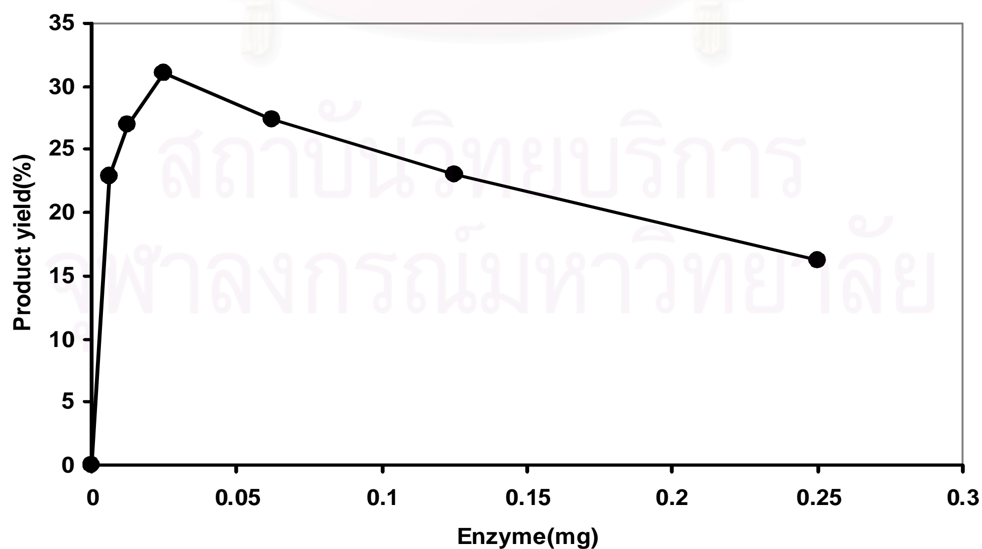

Figure 21 Effect of amount of enzyme on oligosaccharide synthesis.

3.4.3 Effect of pH

The effect of pH on oligosaccharide synthesis was determined as described. The buffers used were : citrate buffer pH 3-5, acetate buffer pH 5-6 and phosphate buffer pH 6-8, mixed with TEP as 50% co-solvent. The reactions were performed by incubating 0.3 M lactose with 0.025 mg enzyme in 50%TEP co-solvent at various pHs at 40 °C for 48 hours. The synthesized oligosaccharide was detected by TLC as described.

TLC chromatogram (Figure 22) shows the synthesized oligosaccharides as one main spot at Rf 0.19 (Oligo-I) in all pH tested, higher density was seen around pH 5-6.5 (lane f to j). Also Oligo-II was faintly observed among this pH range. When the density on chromatogram was analyzed by Imaging Densitometer and interpreted as percent product yield (Figure 23), the optimum pH which gave the maximum yield of oligosaccharide product (Oligo-I) was citrate buffer pH 4.5.

3.4.4 Effect of temperature

The effect of temperature on oligosaccharide synthesis was determined as described in section 2.9.3. The temperatures used were in the range of 30-70 °C. The reactions were performed by incubating 0.3 M lactose with 0.025 mg enzyme in 50%TEP co-solvent, pH 4.5 at various temperatures for 48 hours. The synthesized oligosaccharide was detected by TLC.

TLC chromatogram (Figure 24) shows the synthesized oligosaccharides as the major Oligo-I and the minor Oligo-II at the temperature around 30-50 °C (lane b to d). At higher temperatures, no spot was clearly seen. When the density on chromatogram was analyzed by Imaging Densitometer and interpreted as percent product yield (Figure 25), the optimum temperature which gave the maximum yield of oligosaccharide product (Oligo-I) was 40°C.

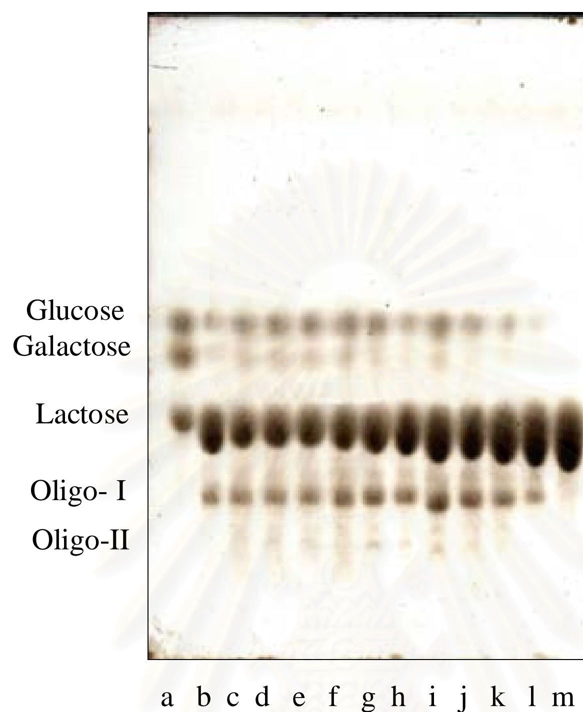

Figure 22 TLC chromatogram of oligosaccharides synthesized at various pHs.

a) Standard glucose, galactose, and lactose (20 µg each)

Synthetic reaction products in, b) citrate buffer pH 3.0; c) citrate buffer pH 3.5; d) citrate buffer pH 4.0; e) citrate buffer pH 4.5; f) citrate buffer pH 5.0; g) acetate buffer pH 6.0; h) acetate buffer pH 6.0; i) phosphate buffer pH 6.0; j) phosphate buffer pH 6.5 k) phosphate buffer pH 7.0; l) phosphate buffer pH 7.5; m) phosphate buffer pH 8.0

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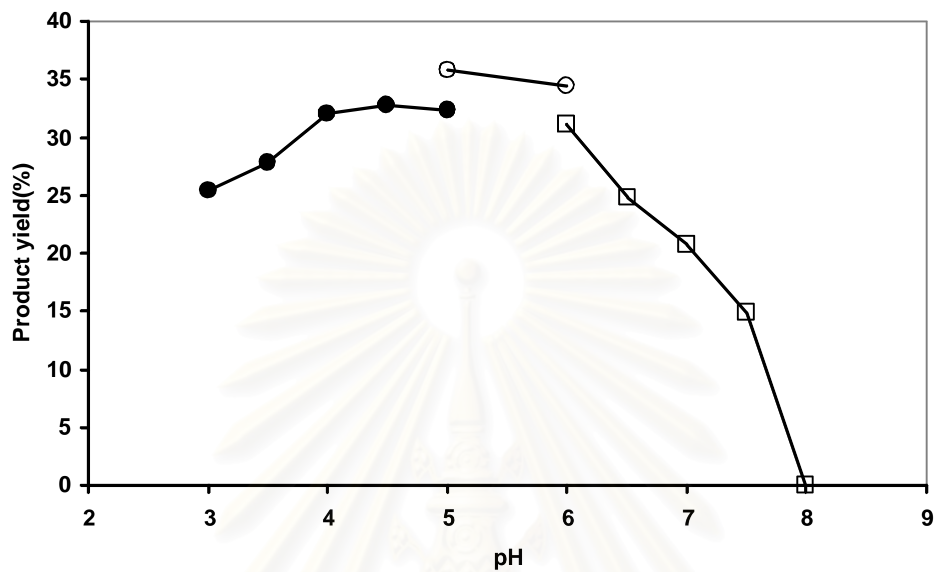

Figure 23 Effect of pH on oligosaccharide synthesis.

Citrate buffer   Acetate Buffer   Phosphate Buffer

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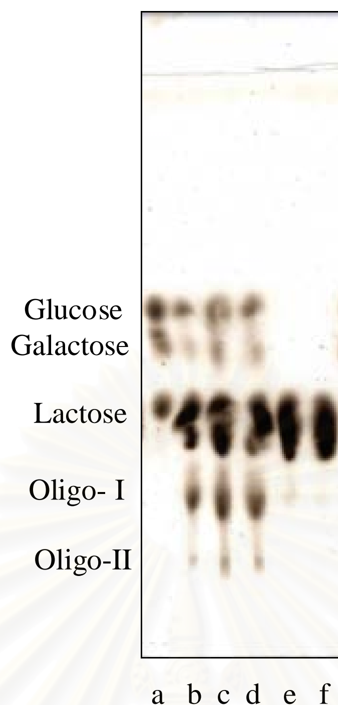

Figure 24 **TLC chromatogram of oligosaccharides synthesized at various temperatures.**

a) Standard glucose, galactose, and lactose (20 µg each)

Synthetic reaction products at, b) 30°C; c) 40°C;

d) 50°C; e) 60°C; f) 70 °C

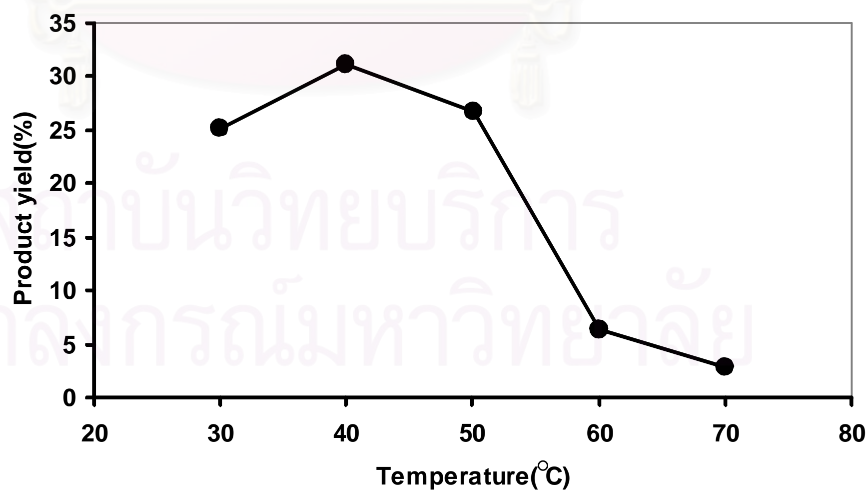

Figure 25 **Effect of temperature on oligosaccharide synthesis.**

3.4.5 Effect of reaction time

The effect of reaction time was determined as described in section 2.9.4. The reaction times used were in the range of 0-96 hours. The reactions were performed by incubating 0.3 M lactose with 0.025 mg enzyme in 50%TEP co-solvent, pH 4.5 at 40 °C for various times. The synthesized oligosaccharide was detected by TLC.

TLC chromatogram (Figure 26) shows both Oligo-I and Oligo-II when incubation time was in the range of 24-72 hours. At less incubation time, only Oligo-I was observed. When the density on chromatogram was analyzed by Imaging Densitometer and interpreted as product yield (Figure 27), the optimum reaction time which gave the maximum yield of oligosaccharide product (Oligo-I) was 36-72 hours.

After optimization, the yield of Oligo-I was 31.6%, an increase of 63.6% and 27.4% when compared with non-optimized condition in the absence and presence of β-cyclodextrin.

3.5 Measurement of fluorescence spectrum of β-galactosidase-β-cyclodextrin co-lyophilizate

To examine the effect of co-lyophilization on the tertiary structure of the enzyme, fluorescence spectrum of β-galactosidase/cyclodextrin complexes were compared with free enzyme. β-galactosidase was lyophilized from citrate buffer pH 4.5 in the presence and absence of cyclodextrin or its derivatives as described in section 2.7. The cyclodextrin and derivatives used were : β-cyclodextrin, hydroxypropyl-β-cyclodextrin and methyl-β-cyclodextrin. The spectrum were determined using excitation wavelength of 280 nm while emission was performed in the range of 290-450 nm.

It was found that the fluorescence emission spectrum of the non-lyophilized, and the lyophilized enzyme in the absence and presence of cyclodextrins were similar. Figure 28 shows the spectra of β-galactosidase-β-cyclodextrin co-lyophilizate compared with free enzyme. Maximum emission wavelength of all spectrum was at 340 nm and fluorescence intensity did not change significantly.

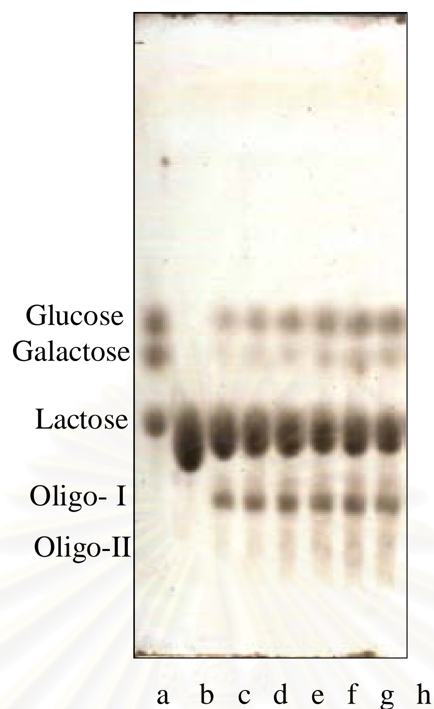

Figure 26 TLC Chromatogram of oligosaccharides synthesized at different reaction time.

a) Standard glucose, galactose, and lactose (20 µg each)

Synthetic reaction products at, b) 0 hr; c) 12 hr; d) 24 hr; e) 36 hr; f) 48 hr; g) 60 hr; h) 72 hr

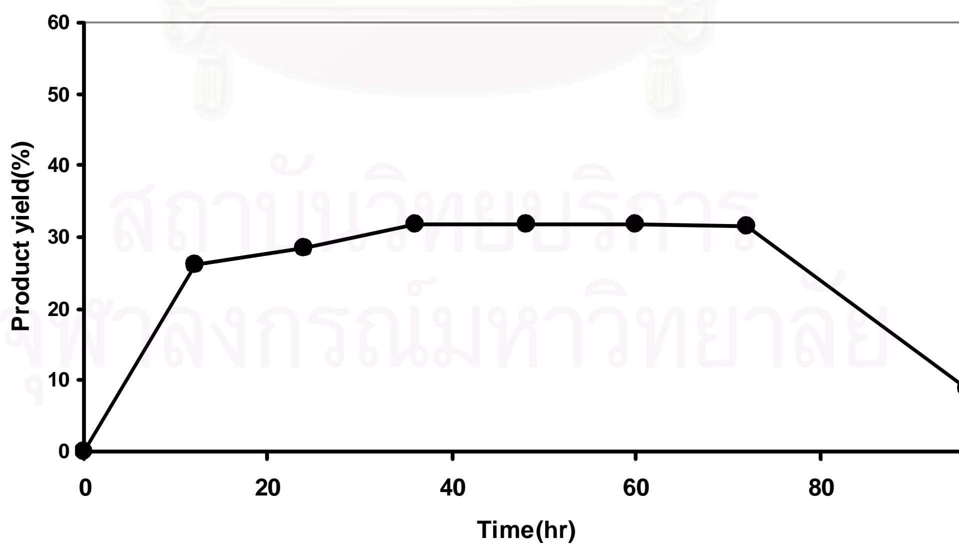

Figure 27 Effect of reaction time on oligosaccharide synthesis.

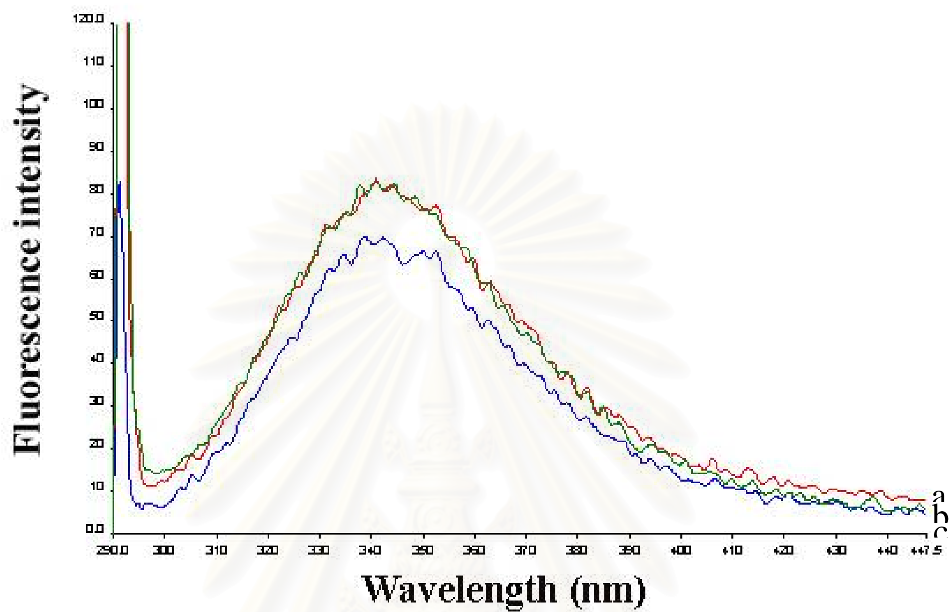

Figure 28 **Fluorescence emission spectrum of β-galactosidase in 50% TEP co-solvent**

a = Non-lyophilized β-galactosidase (red line)

b = Lyophilized β-galactosidase (green line)

c = β-galactosidase/β-cyclodextrin co-lyophilizate (blue line)

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3.6 Fourier transform infrared (FT-IR) spectroscopy

To examine the effect of co-lyophilization with β-cyclodextrin on the secondary structure of β-galactosidase, the fluorescence spectra of β-galactosidase/β-cyclodextrin complex was compared with free enzyme. β-galactosidase was lyophilized from citrate buffer pH 4.5 in the presence and absence of β-cyclodextrin as described in section 2.7.

The result (Figure 29) showed significant spectral change in β-galactosidase upon lyophilization. The spectrum of lyophilized β-galactosidase and β-galactosidase/β-cyclodextrin complex were significantly different from non-lyophilized enzyme, both in Amide I bond region (1600-1700 cm^{-1}) and Amide II bond region (1510-1580 cm^{-1}). However, when compared the co-lyophilized with the control lyophilized (lyophilized in the absence of β-cyclodextrin) enzyme, no significant change in the spectrum was observed.

3.7 Characterization of oligosaccharide products

From TLC chromatograms, the synthesized oligosaccharides show one main spot at Rf 0.19 (named Oligo-I) and one minor spot at Rf 0.10 (named Oligo-II). To characterize these oligosaccharides, the products were scraped from PLC as described in section 2.11.

3.7.1 Mass spectrometry (MS)

Molecular weights of the synthesized oligosaccharides were investigated as described in section 2.12. For Oligo-I, the ESI-TOF MS of this oligosaccharide product exhibited the pseudomolecular ion peak $[M+Na]^+$ at *m/z* 527.15 which was corresponded to the size of a trisaccharide (the calculated value for $C_{18}H_{32}O_{16}$ + Na was at 527.42) (Figure 30). For Oligo-II, the MS spectrum exhibited the pseudomolecular ion peak $[M+Na]^+$ at *m/z* 689.21 which was corresponded to the size of a tetrasaccharide (the calculated value for $C_{24}H_{42}O_{21}$ + Na was at 689.56) (Figure 31). Furthermore, Oligo-II also exhibited the pseudomolecular ion peak

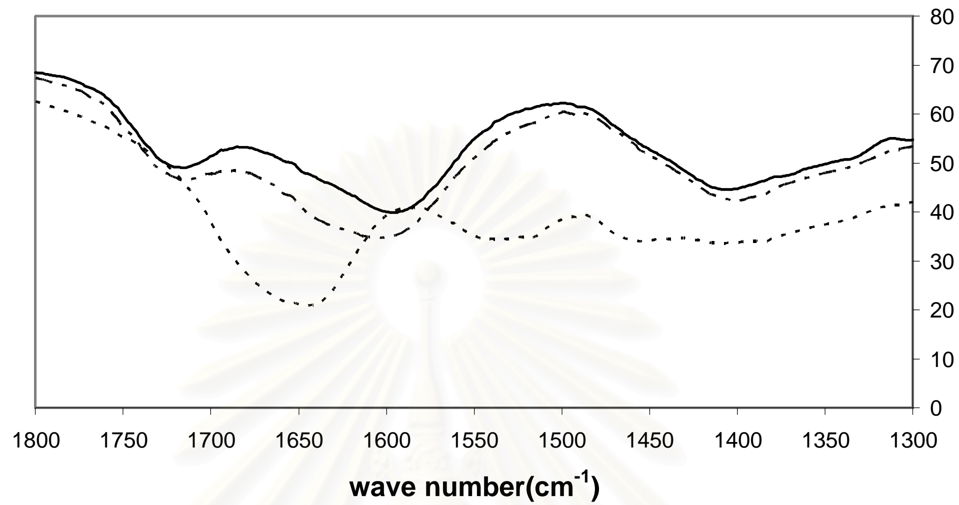

Figure 29 FT-IR spectrum of β-galactosidase

——— β-galactosidase/β-cyclodextrin co-lyophilizate

······· Non-lyophilized β-galactosidase

— · — lyophilized β-galactosidase

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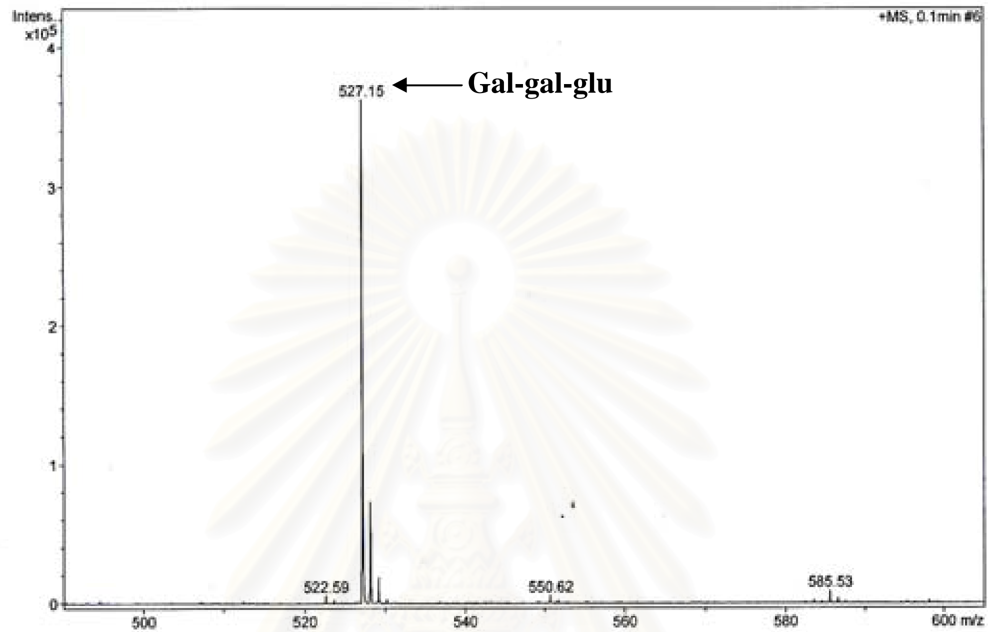

Figure 30 ESI-TOF mass spectra of Oligo-I

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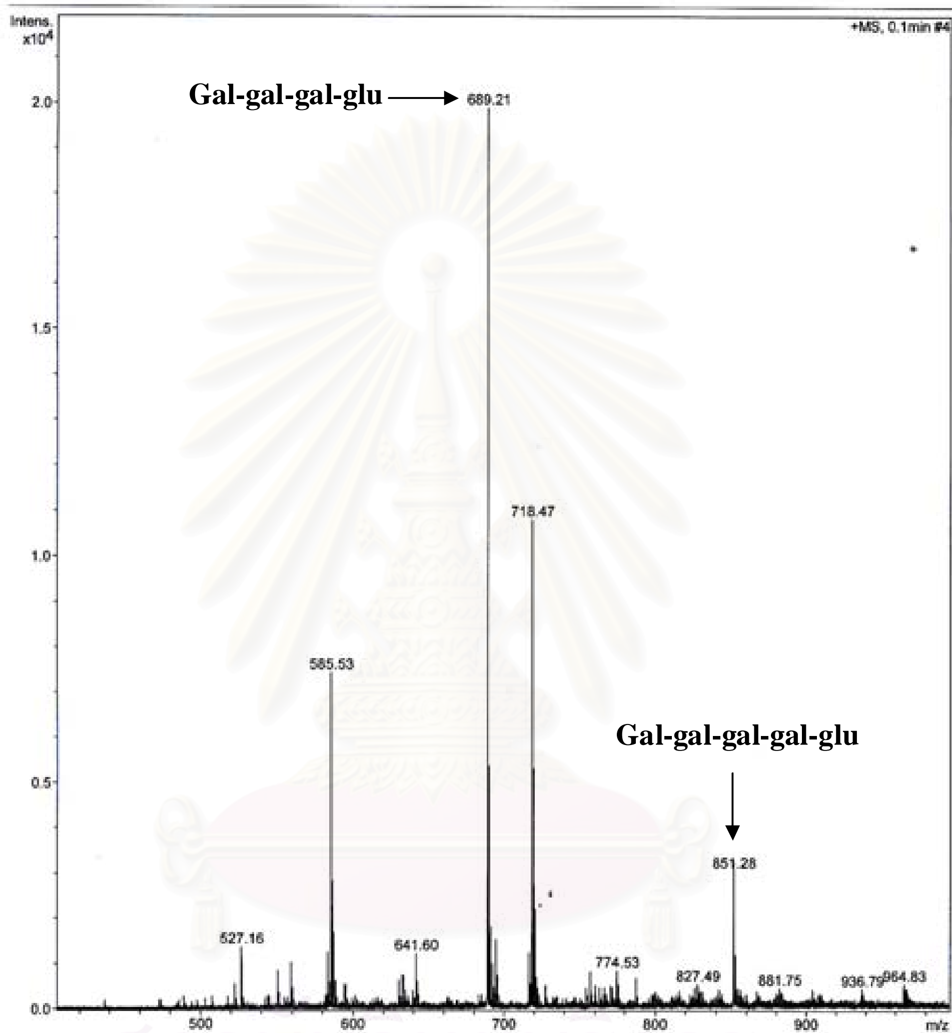

Figure 31 ESI-TOF mass spectra of Oligo-II

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$[M+Na]^+$ at *m/z* 851.28 which was corresponded to the size of a pentasaccharide (the calculated value for $C_{30}H_{52}O_{26}$ + Na was at 851.70) (Figure 31).

3.7.2 Nuclear magnetic resonance (NMR)

For the structural elucidation of Oligo-I and Oligo-II, ^{1}H and ^{13}C NMR were used in order to investigate combining information of the linkage. The one-dimension ^{1}H-NMR of Oligo-I in D_2O (Figure 32 and Table 7) displayed characteristic signal for β-linkage between Gal and Gal at δ = 4.478 ppm (j = 8.1). While the ^{13}C-NMR of Oligo-I in D_2O (Figure 33 and Table 8) displayed characteristic signal of 1-6 linkage between Gal and Gal at δ = 71.365 ppm. From ^{1}H and ^{13}C-NMR analysis, the Oligo-I was determined to be *O*-β-D-galactopyranosyl-(1-6)-*O*-β-D-galactopyranosyl-(1-4)-D-glucopyranose (6'-galactosyl-lactose). For Oligo-II, because of impurities and only low amount available, the NMR spectrum did not give good signal (data not shown).

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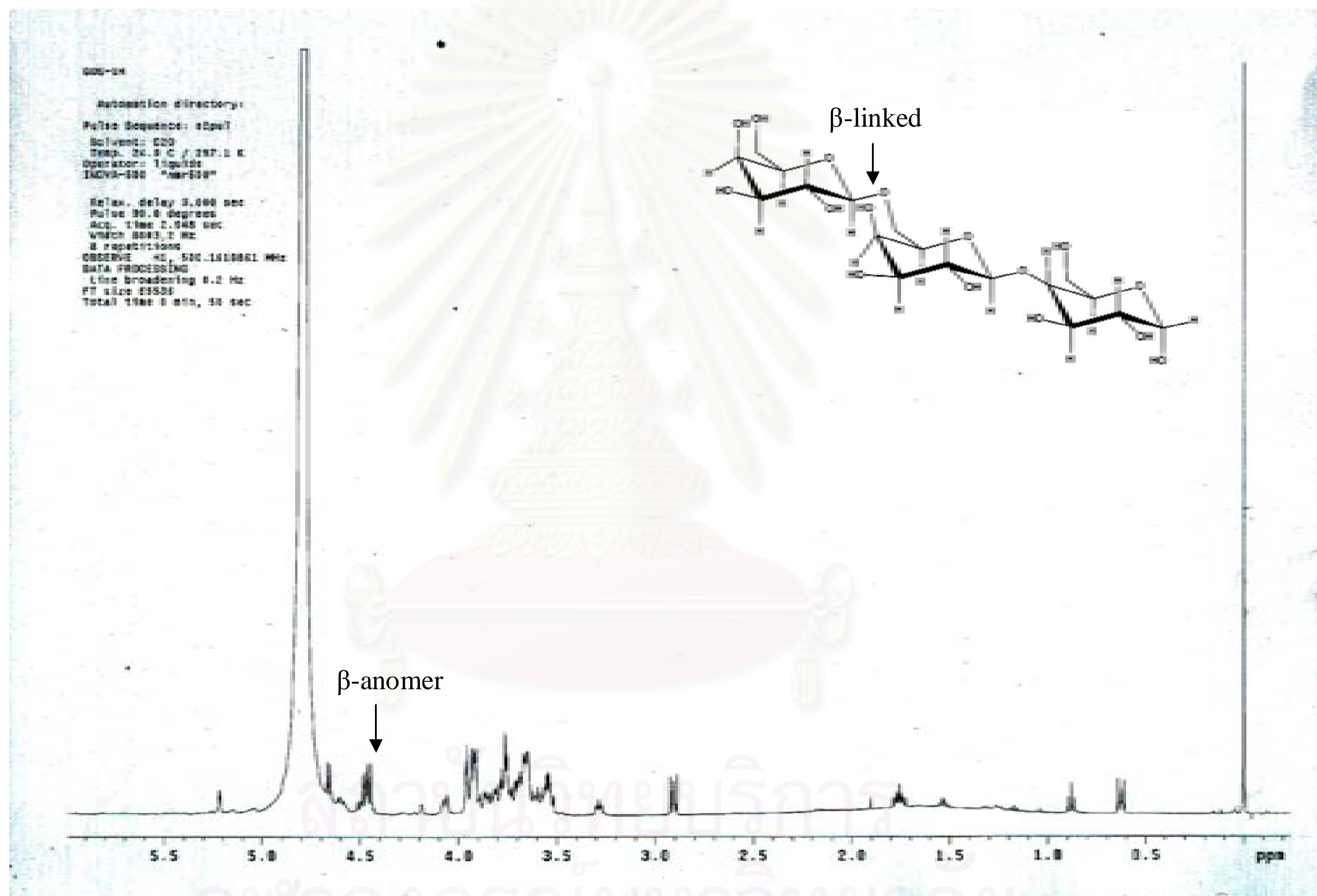

Figure 32 **The 500 MHz ^{1}H-NMR spectra of Oligo-I**

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Table 7 ^{1}H-NMR chemical shifts for Oligo-I (Gal''-Gal'-Glu)

	Chemical shift (ppm)
H-1-α	5.209
H-2-α	3.587
H-3-α	3.903
H-4-α	3.678
H-5-α	3.826
H-6-α-alpha	3.926
H-6-α-beta	3.883
H-1-β	4.660
H-2-β	3.279
H-3-β	3.659
H-4-β	3.672
H-5-β	3.643
H-6-β-alpha	3.956
H-6-β-beta	3.860
H-1'	4.462
H-2'	3.579
H-3'	3.694
H-4'	3.950
H-5'	3.808
H-6'-alpha	3.910
H-6'-beta	3.769
H-1''	4.478
H-2''	3.554
H-3''	3.666
H-4''	3.920
H-5''	3.753
H-6''-alpha	3.777
H-6''-beta	3.792

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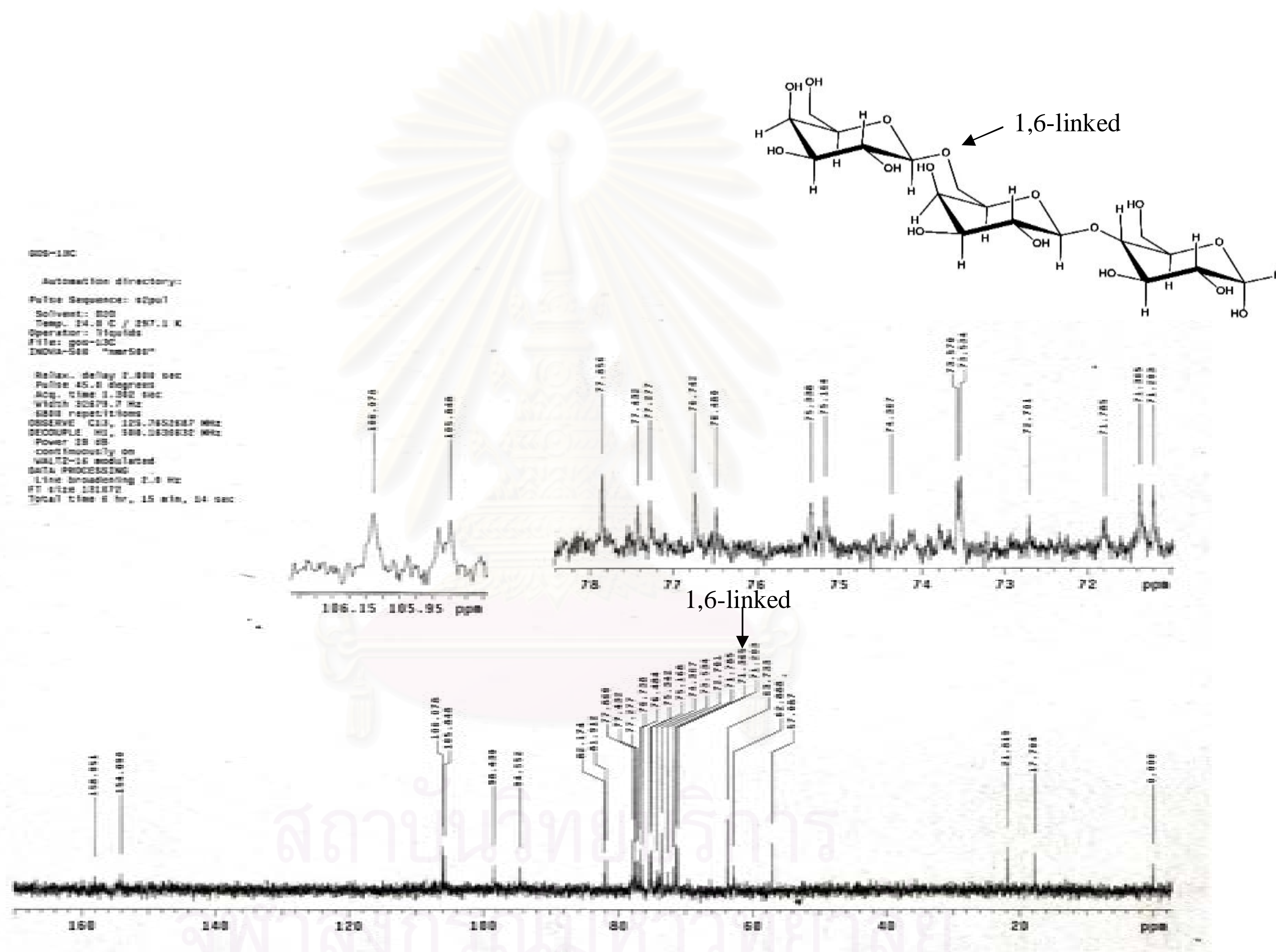

Figure 33 The 125 MHz ^{13}C-NMR spectra of Oligo-I

Table 8 ^{13}C-NMR chemical shifts for Oligo-I (Gal''-Gal'-Glu)

	Chemical shift (ppm)
C-1-α	94.552
C-2-α	75.168
C-3-α	73.534
C-4-α	82.174
C-5-α	72.701
C-6-α	73.534
C-1-β	98.430
C-2-β	76.484
C-3-β	76.738
C-4-β	81.912
C-5-β	77.432
C-6-β	62.888
C-1'	105.848
C-2'	71.203
C-3'	75.168
C-4'	71.785
C-5'	77.860
C-6'	71.365
C-1''	106.078
C-2''	74.367
C-3''	75.342
C-4''	71.203
C-5''	77.277
C-6''	63.733

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CHAPTER IV

DISCUSSION

4.1 Selection of most appropriate organic solvent for synthetic reaction of β-galactosidase

Enzymatic catalysis under organic solvent conditions has become an important aspect. Many reasons have favored the use of enzyme in organic solvent rather than aqueous solution. In an organic reaction condition, enzymes show several interesting properties such as: enhanced stability, solubility of nonpolar substrate and stereospecificities (Klibanov, 1997; Griebenow et al., 1999; Klibanov, 2001). Furthermore, enzyme selectivity in organic solvent is not only distinct from that in aqueous but can be markedly controlled, and even reversed (Klibanov, 1997; Santos et al., 1999; Klibanov, 2001).

β-galactosidase (EC 3.2.1.23) is one of the enzymes in hydrolase group which can activate hydrolysis of glycosidic bonds in carbohydrates that consist of lactose (Huber et al., 1976). However, under certain conditions, this enzyme also catalyzes the synthesis of oligosaccharides. Oligosaccharides production in organic solvent has special advantages because the thermodynamic equilibrium can be shifted to synthetic direction by reversing the normal hydrolysis owing to the limitation of water activity in the reaction system (Crout and Vic, 1998; Chen et al., 2001). Other strategy which was widely used in oligosaccharide synthesis is transglycosylation. In this case, enzyme catalyzes to transfer glycosyl moiety to accepter, and yield depends on the rate of product formation relative to the rate of hydrolysis (Monsan and Paul, 1995). Oligosaccharide production from lactose with β-galactosidases from *Aspergillus oryzae* (Iwasaki et al., 1996; Ismail et al., 1999; Reuter et al., 1999; Gaur et al., 2006), *Escherichia coli* (Reuter et al., 1999; Chen et al., 2001; Chen et al., 2003), *Bifidobacterium infantis* HL96 (Hung et al., 2001), *Penicillium simplicissimum* (Cruz et al., 1999), *Sulfolobus solfataricus* (Reuter et al., 1999) and *Sirobasidium magnum* (Onishi and Tanaka, 1997) has been reported. However, most enzymes used in these studies are not approved for food use and are expensive. Furthermore, many of them are from the source not commercially available. Among them, *Aspergillus oryzae* β-galactosidase is already used in food products, and is

relatively inexpensive as compared to enzyme from other sources. From these reasons, β-galactosidase from *Aspergillus oryzae* was chosen for developing efficiency in oligosaccharide production by immobilization in celite or chitosan (Gaur et al., 2006), or in reverse micelle (Chen et al., 2001; Chen et al., 2003) or by media optimization (Ismail et al., 1999; Chen et al., 2001; Isabel del-Val and Otero, 2003). In our work, media optimization by using organic solvent for reducing water content in reaction was used for developing oligosaccharide production of β-galactosidases from *Aspergillus oryzae*.

The commercial *A.oryzae* β-galactosidase used in our study is highly pure since only one band was observed on SDS-PAGE (Figure 6). Molecular weight of this enzyme estimated from its Rf value is approximately about 55 kD. The native form has been reported to be about 105-112 kD (Tanaka et al., 1975, Mega and Matsushima, 1979), thus this enzyme is composed of two subunits of the same size, like the β-glucosidase of *Aspergillus fumigatus* (Rudick and Elbein, 1975) and the β-galactosidase of *Cicer arietinum* L (Wolfgang and Wolfgang, 1975).

The organic solvents which were used in this study are water miscible and have been previously reported as being co-solvents that could retain significant activity of β-galactosidase during extended periods of incubation. In 1992, Laroute and Willemot reported the influence of solvents on enzymatic stability and activity of glucoamylase and β-galactosidase. 1,4-butanediol and 1,5-pentanediol were solvents (used as 99% and 97%, respectively) which could retain significant activity of β-galactosidase after 24 hours incubation at 24 °C. In 2005, Yoon and Mackenzie examined β-galactosidase activity in organic solvents. Their results suggested that methoxyethyl acetate and triethyl phosphate could retain good hydrolytic enzyme activity. Thus, organic solvents chosen for our study were: 1,4-butanediol, 1,5-pentanediol, methoxyethyl acetate (MEA), triethyl phosphate (TEP), and acetonitrile. Synthetic activity of β-galactosidase was investigated in various organic solvent-citrate buffer mixtures (50% v/v citrate, pH 4.5) with a view to finding solvent systems of reduced water content suitable for synthesis of galacto-oligosaccharides. It has been reported that the ratio of organic solvents could markedly influence on catalytic efficiency of enzymes. In 2003, Hasegawa and colleagues studied the reaction calatyzed by α-chymotrypsin/cyclodextrin complex in acetonitrile using *N*-acetyl-L-tyrosine ethyl ester (ATEE) substrate. They reported that percent water content had significant effect on the yield and reaction rate of both transesterification and hydrolysis. In 1999, Ismail and colleagues synthesized butylgalactoside from *A.oryzae*

β-galactosidase in butanol. They demonstrated that water content in reaction had significant effect on oligosaccharide synthesis. Our study thus varied solvent ratio to obtain best result.

The criteria used for selection of the appropriate organic solvent for β-galactosidase was to find solvent system which could retain good hydrolytic activity and suitable for the synthesis of oligosaccharides. The high efficiency of the enzyme in oligosaccharide production was the key factor in judging the system.

First, the hydrolysis activity of β-galactosidase was determined in various organic solvents using different ratios of solvent to citrate buffer since emulsion was obtained at high proportion of some organic solvents which made activity measurement by absorbance impossible. The results (Figure 7) demonstrated that the ratio of organic solvent had significant effect on hydrolysis activity of β-galactosidase. The higher the ratio, the lower the hydrolysis activity. Similar situation was reported for α-chymotrypsin (Simon et al., 1998; Hasegawa et al., 2003), β-galactosidase (Ismail et al., 1999; Chen et al., 2001), pancreas trypsin (Simon et al., 1998); carboxypeptidase A (Simon et al., 1998), and porcine pancreas lipase (Simon et al., 1998). In our study, by using the maximum ratio of each solvent, 84-92% of the activity in relative to the activity in citrate buffer was found with 20%TEP, 50%MEA, and 40% acetonitrile co-solvents, respectively. The ratios used in the next step was the maximum ratios except for 1,4-butanediol and 1,5-pentanediol which are the most polar, 50:50 (v/v) was used.

Then, for oligosaccharide synthesis, all solvents were used in the same ratio, as 50% co-solvents. The result analyzed by TLC (Figure 8) shows that only in citrate buffer and co-solvents with MEA and TEP, the synthesized oligosaccharide (named Oligo-I) was observed. Fifty percentage TEP co-solvent gave the highest amount of oligosaccharide. It was interesting to note that acetonitrile which gave rather high hydrolysis activity was not good for oligosaccharide synthesis. These results agree well with previous studies. In 1997, Finch and Yoon synthesized galactose disaccharides in the presence of 67% TEP co-solvent using β-galactosidase from *Kluyveromyces fragilis* and 60% TEP using β-galactosidase from *Bacillus circulans* and *Diplococcus pneumoniae*. They reported that the 67% acetonitrile co-solvent which conferred a high retention of hydrolytic stability on the *K. fragilis* enzyme or 77% MEA for *A. oryzae* enzyme did not necessarily aid the synthesis of disaccharides. In 67% TEP, the media in which the activity of β-galactosidase from both sources was reasonably stable, the degree of disaccharide synthesis was high. The reason for this behavior is not immediately obvious,

and it appears that solvents can have different effects on different reactions catalyzed by the same enzyme.

MEA and TEP were chosen for varying solvent ratios and determined for oligosaccharide synthesis. Two ratios, 50:50 and 20:80 (organic solvent : buffer) (v/v), were used. The oligosaccharide product was detected by both TLC (Figure 9) and HPLC. Oligo-I was observed in all solvents on TLC plates. The spot intensity of Oligo-I produced in different solvents was in the order of, 50%TEP > 20%TEP > 50%MEA > 20%MEA. The overall results demonstrated that β-galactosidase catalysis is markedly affected by different co-solvents. It is suggested that, in addition to the effect on kinetics and thermodynamics of the reaction, co-solvents may perturb the conformational stabilities of the enzyme in several ways, including disturbing the hydration layers essential for enzyme catalysis and proper folding, as well as alterations to the protein structure by direct interactions with protein solvation sites, either hydrophobic or H-bonding (Yoon and Mckenzie, 2005).

When amount of Oligo-I was determined by HPLC (Figure 10), the solvent peak was at 3.1 minutes while the peak at 4.6 minutes contained galactose and glucose. Oligo-I was eluted at 8.0 minutes, after lactose at 6.1 minutes, suggesting that it might be a trisaccharide. Synthesis of Oligo-I in 50% TEP solution gave 1.6 and 6.8 times higher product yield than in citrate buffer and 50% MEA solution respectively. However, the yield obtained in TEP co-solvent was only 18.5% (Figure 13). In 2005, Yoon and Mckenzie have reported that for the *A. oryzae* β-galactosidase, best hydrolytic activity is in MEA, followed by trimethyl phosphate and TEP, using 50% v/v acetate buffer, pH 4.5, but for the synthesis of disaccharides, the best yield of product is in TEP. Although this report did not give the actual value of the product yield, the trend is in agreement with our results.

In the attempt to increase oligosaccharide synthesis by reducing water activity of the reaction mixture, the enzyme was lyophilized, then suspended in smaller amount of buffer or co-solvent. The hydrolysis activity of lyophilized and non-lyophilized enzyme was compared. The activity was decreased approximately 45.5% upon lyophilization. This result agrees with the study of Izutsu and colleagues which reported that after lyophilization, hydrolysis activity of β-galactosidase from *A. oryzae* decreased (Izutsu et al., 1993). In contrast, in the oligosaccharide synthetic reaction, we found some increase in oligosaccharide synthesized by the lyophilized enzyme, especially when synthesis was

in aqueous solution (Figure 13). The product yields were 13.6% and 11.1% for the lyophilized and non-lyophilized enzyme, in the aqueous buffer synthesis system. However, the same product yield, 18.5% was obtained for both lyophilized and non-lyophilized enzyme in 50% TEP co-solvent. Ooe et al. showed that transesterification and hydrolysis reaction of lyophilized α-chymotrypsin in 97% acetonitrile was greater than non-lyophilized enzyme (Ooe et al., 1999).

From these results, 50%TEP-co-solvent is the appropriate solvent system chosen. Although this system cannot be used for determination of hydrolysis activity by lyophilized and non-lyophilized β-galactosidase, the amount of oligosaccharide synthesized is the major factor in choosing the type and ratio of organic solvent to be used in the next step.

4.2 Effect of cyclodextrins on oligosaccharide synthesis by β-galactosidase in the presence of organic solvent

In our work, the possibility of improving synthetic activity of β-galactosidase in co-solvents by using the enzyme co-lyophilized with cyclodextrins was explored. Co-lyophilization of the enzyme from an aqueous solution of 50 mM citrate buffer, pH 4.5 with the addition of β-cyclodextrin, its derivatives: hydroxypropyl-and methyl-, and short-chain oligosaccharides: G_5 and G_7, was performed. These β-galactosidase-oligosacharide co-lyophilizate enzymes at 1:5 w/w were used for oligosaccharide synthesis in 50% TEP in comparison with soluble (non-lyophilized) and lyophilized enzyme prepared in the absence of excipients. Percent product yields of Oligo-I (Figure 15) showed that lyophilization had no effect on synthetic activity of the enzyme in 50%TEP co-solvent, as the non-lyophilized and lyophilized enzyme gave product yield of approximately 19 %. All oligosaccharides used, either linear or cyclic, were able to increase product yield to a similar extent. This result was different from the study in chymotrypsin, which demonstrated that glucose, maltose, and dextrin had negative effect on enzyme catalysis, only the cyclodextrins demonstrated large positive effect (Hasegawa et al., 2003). Among cyclodextrins, hydroxypropyl derivative had a larger positive effect than its parent, the beta-form, and the other native cyclodextrins showed different extents of catalysis improvement. However, in comparison of catalysis improvement, kcat/Km values were used in their study while product yields were used in ours. From our result, β-

cyclodextrin had the largest effect. The observed increase in oligosaccharide synthesis from the β-galactosidase-β-cyclodextrin co-lyophilizate, though marginal, was 20.9% when compared with synthesis from lyophilized or non-lyophilized enzyme.

It is known that sugar is one of well-known cryoprotectants and polyhydroxy compounds which can retain stability while lyophilization (Izutsu et al., 1993; Izutsu et al., 2004, Hudson et al., 2005). For cyclodextrin, in addition to retaining catalytic activity, it also improved catalytic activity in organic solvent. The various effects of organic solvent are following: the partial loss of tertiary and secondary structure, solvent penetration into the active site, and the rigidification of enzyme conformation (Hudson et al., 2005). The enzyme in organic solvent is rigid because organic solvent lack water's ability to engage in multiple hydrogen bonds, and also have lower dielectric constants, leading to stronger intraprotein electrostatic interaction (Klibanov, 2001). Cyclodextrins was reported as "molecular lubricant", the more flexible the enzyme is, the greater the probability that the enzyme achieves a more active conformation (Griebenow et al., 1999; Hudson et al., 2005).

Studies on the improvement of catalytic activity of proteases and lipases in organic solvents by co-lyophilization of the enzymes with cyclodextrins have been reported. In 1999, Griebenow et al. demonstrated that transesterification in anhydrous tetrahydrofuran catalyzed by subtilisin Carlsberg co-lyophilized with methyl-β-cyclodextrin at 1:6 w/w was increased by up to 164-fold and the enantioselectivity was doubled. In another report, α-chymotrypsin co-lyophilized with hydroxypropyl-β-cyclodextrin at 1:4 w/w displayed much higher activity than free chymotrypsin for transesterification in acetonitrile/water (97:3, v/v). The activity increased with an increase in water content in the reaction media, and the maximum activity was obtained at 5-10% water (Hasegawa et al., 2003). For lipase, Mine et al.,2003, demonstrated a 17-fold increase in initial rate of transesterification and also an increase in enantioselectivity by lipase-permethylate-β-cyclodextrin co-lyophilizate. These reports suggest that the interaction between cyclodextrin and the enzyme could help stabilize the enzyme structure during lyophilization, thus provides a more active and enantioselective conformation. Cyclodextrins thus resemble crown ether, which has been successively used to activate enzymes by increasing flexibility in solvents as a result of co-lyophilization (Griebenow et al., 1999).

Type of cyclodextrin appropriate for each enzyme is different, such as methyl-β-cyclodextrin for subtilisin Carlsberg (Griebenow et al., 1999; Santos et al., 1999;

Montańez-Clemente et al., 2002), 2,3,6-tri-*O*-methyl-β-cyclodextrin and hydroxypropyl-β-cyclodextrin for α-chymotrypsin (Ooe et al., 1999, Hasegawa et al., 2003), peracetylated β-cyclodextrin and permethylated β-cyclodextrin used for *Pseudomonas cepacia* lipase (Ghanem and Schurig,2001, Mine et al., 2003), and hydroxypropyl β-cyclodextrin for *Rhizopus niveus* lipase (Ávila-González et al., 2005). These results suggested that various types of cyclodextrin could make various effects to enzymes. For enzyme in the glycosidase group, no previous study using cyclodextrin for the improvement of enzyme catalysis has been reported.

The ratio of β-galactosidase / β-cyclodextrin complex markedly influenced catalytic reaction (Griebenow et al., 1999; Ghanem and Schurig, 2001; Hasegawa et al., 2003). The result (Figure 16 and 17) showed that β-cyclodextrin : β-galactosidase ratios of 5:1 and 10:1 gave the highest yield. For the higher ratio, synthesis activity was about the same level or even lower when compared to that of lyophilized β-galactosidase in the absence of β-cyclodextrin. This result corresponds with data previously presented that the ratio of chymotrypsin to hydroxypropyl β-cyclodextrin had a significant effect on catalytic activity of transesterification reaction (Hasegawa et al., 2003).

4.3 Optimization of oligosaccharide synthesis

In order to produce higher yields of oligosaccharides, the synthesis condition in 50%TEP was optimized. Parameters optimized were: substrate (lactose) concentration, amount of enzyme, pH, temperature and reaction time.

When lactose concentration was varied from 0-0.3 M, (Figure 18 and 19), maximum product yield was obtained at 0.3 M. This optimal substrate concentration was lower than concentration used in aqueous solution previously reported (Iwasaki et al., 1999; Chen et al., 2001; Aslan and Tanriseven, 2007). Because of limitation of solubility of substrate in organic solvent, lactose concentration higher than 0.3 M could not be prepared in 50% TEP co-solvent. If the concentration is more than 0.3 M, substrate will recrystallize.

For pH effect, slight shifts in the pH from the optimum value lead to a decrease in the reaction rate, because of changes in the ionization of charged amino acid residues that function in the active site of the enzyme. However, large shifts in pH lead to denaturation of the enzyme because of interference with many weak noncovalent bonds maintaining its three-dimensional structure (Segal, 1976). The result (Figure 22 and 23) showed that

optimum pH which gave the maximum yield of oligosaccharide product was pH 4.5 in citrate buffer. This result agrees with previous studies (Ismail et al., 1999; Gaur et al., 2006). Moreover, pH 4.5 is optimal for hydrolysis activity of this enzyme (Tanaka et al., 1975; Gaur et al., 2006). When temperature was varied, maximum yield of oligosaccharide product was at 40°C (Figure 24 and 25). This result is different from optimum temperature of enzyme in aqueous solution which was reported to be 50-55 °C (Ismail et al., 1999; Gaur et al., 2006). The difference of optimum temperature should be due to the presence of organic solvent and the use of enzyme co-lyophilized with β-cyclodextrin in this study. The result of our work corresponded to that reported by Mine and colleagues (2003). They showed that the temperature which made the highest enzyme activity of *Pseudomonas cepacia lipase* in diisopropyl ether of enzyme complex with permethylated β-cyclodextrin was lower than native enzyme.

The optimal condition obtained was with 0.3 M lactose incubated with 0.025 mg (β-galactosidase-β-cyclodextrin 1:5 co-lyophilizate) enzyme at pH 4.5, 40℃ for 36-72 hours. After optimization, in addition to a major Oligo-I product, a minor Oligo-II was also observed on TLC plates. In determination of percent product yield, only the yield of Oligo-I, the major product, was calculated. The product yield after optimization was 31.6%, an increase of 63.6% and 27.4% when compared with non-optimized condition in the absence and presence of β-cyclodextrin. Several reports on oligosaccharide synthesis using different β-galactosidase and synthesis condition gave different percent product yield. In 1995, Onishi and colleagues reported 64% yield of galacto-oligosaccharide from 360 mg/ml lactose catalyzed by enzyme of *Sterigmatomyces elviae* CBS8119. By using immobilized enzyme, 17.3% and 15.8% yields of galacto-oligosaccharides were reported from 20% and 30% lactose catalyzed by chitosan-immobilized *A. oryzae* and immobilized Pectinex Ultra SP-L, respectively (Gaur et al., 2006; Aslan and Tanriseven, 2007).

4.4 Investigation of the change in secondary and tertiary structures of β -galactosidase upon co-lyophilization with β-cyclodextrin

To examine the effect of co-lyophilization with β-cyclodextrin on the secondary and tertiary structures of β-galactosidase, the fluorescence and FT-IR spectrum of β-galactosidase/β-cyclodextrin complex were compared with free enzyme. It was found that fluorescence emission spectrum of the free enzyme in solution (non-lyophilized), the

lyophilized enzyme in the absence and presence of cyclodextrins were similar (Figure 28). Maximum emission wavelength of aromatic amino acids, especially tryptophan, at 340 nm was observed in all cases. This result suggested that interaction of cyclodextrins with enzyme did not affect the environment around tryptophan. When FT-IR spectrum were examined, lyophilization resulted in significant change in Amide I (1600-1700 cm^{-1}) and Amide II (1510-1580 cm^{-1}) bond regions (Figure 29). However, co-lyophilized with β-cyclodextrin had no effect on the spectrum of the enzyme. Griebenow and colleagues (1999) determined FT-IR spectrum of subtilisin co-lyophilized with MβCD under various organic solvents, they reported that significant solvent-induced structural perturbations in the MβCD co-lyophilizate, especially in the Amide I bond region was observed in toluene, dichloromethane and acetonitrile. However, no change in FT-IR spectra was obtained when the co-lyophilizate was performed in tetrahydrofuran, dioxane and octane (all solvents used as anhydrous form). Thus, solvent plays a critical role in preservation of secondary structure of enzyme.

Measurement of fluorescense spectrum of tryptophan is commonly used as a means to follow a change in tertiary structure of proteins or enzymes since surface tryptophan residues are usual target for interactions with solvent or other molecules (Copeland, 1994; Yu et al., 2001). In addition, Amide bond regions have been used to characterize stretching vibration of peptide carbonyl (C=O) (Amide I bond region) and amide (N-H) (Amide II bond region) of enzymes, spectrum obtained are useful for prediction of protein secondary structure (Pelton, 2001). Though β-cyclodextrin did not cause a change in tryptophan environment and Amide bond regions of β-galactosidase-β-cyclodextrin co-lyophilizate, the result did not exclude the overall change in three dimensional structure of the enzyme due to H-bonding, ionic, or hydrophobic interaction between cyclodextrins and functional groups on enzyme.

4.5 Characterization of oligosaccharide products

From TLC chromatograms, the synthesized oligosaccharides show one main spot at Rf 0.19 (as Oligo-I) and one minor spot at Rf 0.10 (as Oligo-II). By ESI-TOF**,** molecular weight of Oligo-I (Figure 30) is 504 (m/z of 527) using the positive mode with Na^+ added. This result suggests that Oligo-I could be a trisaccharide. Oligo-II (Figure 31) was a mixture of molecular weight of 666 (m/z 689) and 828 (m/z 851), which suggests

that Oligo-II may compose of 2 types of sugars, as tetra- and pentasaccharides (Figure 31). The other two peaks at m/z of 585.53 and 718.47 were not found in negative mode of analysis (data not shown), these peaks might be the result of interaction of sodium, not the product peaks.

Both Oligo-I and II were analyzed by ^{1}H and ^{13}C NMR for structural elucidation. For configuration analysis, the ^{1}H-NMR spectrum of Oligo-I was obtained and compared with ^{1}H-NMR spectrum of lactose (Appendix 6). The H-1" of Oligo-I was found at δ = 4.478 ppm and j = 8.1 Hz. When compared with the chemical shifts of previous reports (Van Laere et al., 1999; Isabel del-Val and Otero, 2003; Lie et al., 2003), the signals were identical with β configuration. Then, from ^{13}C-NMR spectrum, the C-6' of lactose subtrate was found at δ = 61.9 ppm. The result from ^{13}C-NMR of Oligo-I suggests the linkage to other residue since C-6' of Oligo-I was found at δ = 69.365 ppm. Therefore, Oligo-I was assigned as 6'-galactosyl lactose (*O*-β-D-galactopyranosyl-(1-6)-*O*-β-D-galactopyranosyl-(1-4)-D-glucopyranose). Our result is similar to previous studies (Reuter et al., 1999; Sako et al., 1999; Aslan and Tenriseven, 2007) which reported that galacto-oligosaccharide synthesized from β-galactosidase of *A. oryzae* preferred β-1,6 linkages. However, other linkages might also occur e.g β-1,4 and β-1,3 (Reuter et al., 1999).

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CHAPTER V

CONCLUSIONS

1. Synthesis of an oligosaccharide by *Aspergillus oryzae* β-galactosidase in 50% TEP co-solvent gave 1.6 times higher product yield than in citrate buffer. The obtained yield was 18.5%.
2. Lyophilization causes significant loss of hydrolysis activity of β-galactosidase while has no effect on oligosaccharide synthesis.
3. Synthetic activity of β-galactosidase in 50% TEP co-solvent was increased significantly by co-lyophilization of the enzyme with β-cyclodextrin. β-galactosidase-β-cyclodextrin co-lyophilizate at 1:5 w/w could increase the oligosaccharide synthesized in 50% TEP by 20.9% when compared with synthesis from lyophilized or non-lyophilized enzyme.
4. The optimal condition for oligosaccharide synthesis in 50% TEP using the β-galactosidase-β-CD co-lyophilizate was 0.3 M lactose incubated with 0.025 mg enzyme at pH 4.5, 40 °C for 36-72 hours. Yield of the major oligosaccharide (Oligo-I) after optimization was 31.6%, an increase of 63.6% over the non-optimized condition in the absence of β-CD.
5. β-CD causes no change in tryptophan fluorescence spectrum and in the Amide bond region of FT-IR spectrum of the enzyme.
6. The major oligosaccharide synthesized a trisaccharide. A mixture of tetra- and penta- galacto-oligosaccharide was also detected.
7. The trisaccharide product was identified as *O*-β-D-galactopyranosyl-(1-6)-*O*-β-D-galactopyranosyl-(1-4)-D-glucopyranose.

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REFERENCES

Aslan, Y. and Tanriseven, A. (2007). Immobilization of Pectinex Ultra SP-L to produce galactooligosaccharides. *Journal of Molecular Catalysis B: Enzymatic*. 45, 73-77.

Ávila-González, R., Pérez-Gilabert, M. and Garcia-Carmoni, F. (2005). Lipase-catalyzed preparation of S-propranolol in presence of hydroxypropyl β-cyclodextrins. *Journal of Bioscience and Bioengineering*. 100(4), 423-428.

Berg, J.M., Tymoczko, J.L., Stryer, L. (2002). Biochemistry. 5th edition. W.H. freeman and company. New York. pp. 301-302.

Bollag, M.D. and Edelstein, J.S. (1991). Protein Methods. A John Wiley & Sons, Inc. New York. pp. 95-142.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry*. 72, 248-254.

Chen, C.W., Ou-Yang, C. and Yeh, C. (2003). Synthesis of galactooligosaccharides and transgalactosylation modeling in reverse micelles. *Enzyme and Microbial Technology*. 33, 497-507.

Chen, S., Wei, D. and Hu, Z. (2001). Synthesis of galacto-oligosaccharide in AOT/isooctane reverse micelles by β-galactosidase. *Journal of molecular catalysis B: Enzymatic*. 16, 109-114.

Copeland, R.A. (1994). Methods of Protein Analysis : a practical guide to laboratory protocol. Chapman & Hall. New York. pp. 179-186.

Crittenden, R.G. and Playne, M.J. (1996). Production, properties and applications of food-grade oligosaccharides. *Trends in Food Science & Technology*. 7, 353-361.

Crout, D.H.G. and Vic, G. (1998). Glycosidases and glycosyl transferases in glycoside and oligosaccharide synthesis. *Current opinion in Chemical Biology*. 2, 98-111.

Cruz, R., Cruz, V.D., Belote, J.G., Khenayfes, M.O., Dorta, C., Oliveira, L.H.S., Ardiles, E. and Galli, A. (1999). Production of transgalactosylated oligosaccharides (TOS) by galactosyltransferase activity from *Penicillium simplicissimun*. *Bioresource Technology*. 70, 165-171.

Cummings, J.H., Gibson, G.R. and Macfarlane, G.T. (1989). Quantitative estimates of fermentation in the hind gut of man. *Acta veterinaria Scandinavica. Supplementum*. 86, 76-82.

Cumming, J.H. and Roberfroid, M.B. (1997). A new look at dietary carbohydrate: chemistry, physiology and health. *European Journal of Clinical Nutrition.* 51,417-423.

Dawson, R.M.C., Elliott, D.C., Elliott, W.H. and Jones, K.M. (1986). Data for biochemical research. 3rd edition. Oxford University Press. New York. pp. 470-478.

Delzenne, N.M. and Roberfroid, M.R. (1994). Physiological effects of non-digestible oligosaccharides. *Lebensmittel-Wissenschaft und-Technologie.* 27, 1-6.

Finch, P. and Yoon, J.H. (1997). The effects of organic solvents on the synthesis of galactose disaccharides using β-galactosidases. *Carbohydrate Research.* 303, 339-345.

Food Navigator. (2001). Cerestar ingredient obtains GRAS status. [cited 2 February 2007]. Available from http://www.foodnavigator.com/news/ng.asp?id= 39991-cerestar-ingredient-obtains.

Gaur, R., Pant, H., Jain, R. and Khare, S.K. (2006). Galacto-oligasaccharide synthesis by immobilized *Aspergillus oryzae* β-galactosidase. *Food chemistry.* 97(3), 426-430.

Ghanem, A. and Schurig, V. (2001). Peracetylated β-cyclodextrin as additive in enzymatic reactions: enhanced reaction rate and enantiomeric ratio in lipase-catalyzed transesterifications in organic solvents. *Tetrahedron: Asymmetry.* 12, 2761-2766.

Giacomini, C., Irazoqui, G., Gonzalez, P., Batista-Viera, F. and Brena, B.M. (2002). Enzymatic synthesis of galactosyl-xylose by *Aspergillus oryzae* β-galactosidase. *Journal of molecular catalysis B: Enzymatic.* 19-20, 159-165.

Gibson, G.R. and Roberfroid, M.B. (1995). Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *The Journal of nutrition.* 125, 1401-1412.

Griebenow, K., Laureano, Y.D., Santos, A.M., Clemente, I.M., Rodriguez, L., Vidal, M.W. and Barlett, G. (1999). Improved enzyme activity and enantioselectivity in organic solvents by methyl-β-cyclodextrin. *Journal of the American Chemical Society.* 121(36), 8157-8163.

Hasegawa, M., Yamamoto, S., Kobayashi, M. and Kise, H. (2003) Catalysis of protease/cyclodextrin complexs in organic solvents effects of reaction conditions

and cyclodextrin structure on catalysis activity of protease. *Enzyme and Microbial Technology*. 32, 356-361.

Huber, R. E., Kurz, G., and Wallenfels, K. (1976). A quantitation of the factors which affect the hydrolase and transgalactosylase activities of β-galactosidase (*E. coli*) on lactose. *Biochemistry*. 15(9), 1994-2001.

Hudson, E.P., Eppler, R.K. and Clark, D.S. (2005). Biocatalysis in semi-aqueous and nearly anhydrous conditions. *Current Opinion in Biotechnology*. 16, 637-643.

Hung, M., Xia, Z. Hu, N. and Lee, B.H. (2001). Molecular and Biochemical analysis of two β-Galactosidase from *Bifidobacterium infantis* HL96. *Applied and Environmental Microbiology*. 67(9), 4256-4263.

Ichikawa, Y., Look, G.C. and Wong, C.H. (1992). Enzyme-catalyzed oligosaccharide synthesis. *Analytical Biochemistry*. 202, 215-238.

Isabel del-Val, M. and Otero, C. (2003). Biphasic aqueous media containing polyethylene glycol for the enzymatic synthesis of oligosaccharides from lactose. *Enzyme and Microbial Technology*. 33, 118-126.

Ismail, A., Soultani, S. and Ghoul, M. (1999). Enzymatic-catalyzed synthesis of alkylglycosides in monophasic and biphasic systems. I. The transglycosylation reaction. *Journal of Biotechnology*. 69, 135-143.

Iwasaki, K., Nakajima, M. and Nakao, S. (1996). Galacto-oligosaccharide production from lactose by enzymic batch reaction using β-galactosidase. *Process Biochemistry*. 31(1), 69-76.

Izutsu, K., Yoshioka, S. and Terao, T. (1993). Stabilization of β-galactosidase by amphiphilic additives during freeze-drying. *International Journal of Pharmaceutics*. 90, 187-194.

Izutsu, K., Aoyagi, N. and Kojima, S. (2004). Protection of protein secondary structure by saccharide of different molecular weights during freeze-drying. Chemical & Pharmaceutical Bulletin. 52(2), 199-203.

Kennedy, J.F. and Pagliuca, G. (1994). Oligosaccharides. In Chaplin, M.F. and Kennedy, J.F. (eds.), *Carbohydrate Analysis*. Oxford University Press. New York. pp. 48-50.

Klibanov, A.M. (1997). Why are enzymes less active in organic solvents than in water? *Trends in Biotechnology*. 15(3), 97-101.

Klibanov, A.M. (2001). Improving enzymes by using them in organic solvents. *Nature*. 409, 241-246.

Laroute, V. and Willemot, R. (1992). Effect of organic solvents on stability of two glycosidases and on glucoamylase-catalysed oligosaccharide synthesis. *Enzyme and microbial technology.*14, 528-534.

Liu, Z., Lu, Y., Zhang, J., Pardee, K. and Wang, P.G. (2003). P1 Trisaccharide (Galα1,4Galβ1,4GlcNAc) synthesis by enzyme glycosylation reactions using recombinant *Escherichia coli. Applied and Environmental Microbiology.* 69(4), 2110-2115.

Loftsson, T. (2005). Cyclodextrin Complexes and Drug Delivery. In Szejtli, J. *Cyclodextrin News.* 18(1), 3, 2004. (Mimeographed)

Maitin, V., Athanasopoulos, V. and Rastall, R.A. (2004). Synthesis of FimH receptor-active manno-oligosaccharides by reverse hydrolysis using α-mannosidase from *Penicillium citrinum, Aspergillus phoenicis* and almond. *Applied microbiology and biotechnology*. 63, 666-671.

Mega, T. and Matsushima, Y. (1979). Comparative studies of three exo-β-glycosidase of *Aspergillus oryzae. Journal of Biochemistry.* 85, 335-341.

Mine, Y., Fukunaga, K., Itoh, K., Yoshimoto, M., Nakao, K. and Sugimura, Y. (2003). Enhanced enzyme acitivity and enantioselectivity of lipases in organic solvents by crown ethers and cyclodextrins. *Journal of Bioscience and Bioengineering.* 95(5), 441-447.

Monsan,P. and Paul, F. (1995). Enzymatic synthesis of oligosaccharides. *FEMS Microbiology Review.* 16, 187-192.

Montańez-Clemente, I, Alvira, E., Macias, M., Ferrer, A., Fonceca, M., Rodriguez, J., Gonzalez, A. and Barletta, G. (2002). Enzyme activation in organic solvents: co-lyopilization of Subtilisin Carlsberg with methyl-β-cyclodextrin renders an enzyme catalyst more active than the cross-linked enzyme crystals. *Biotechnology and Bioengineering.* 78(1), 53-59.

Nakakuki, T. (2002). Present status and future of functional oligosaccharides development in Japan. *Pure and applied chemistry*. 74(7), 1245-1251.

Nelson, D.L. and Cox, M.M. (2000). Lehninger principles of biochemistry. 3rd edition. Worth publishers. New York. pp. 293-302.

Nilsson, K.G.I. (1988). Enzymatic synthesis of oligosaccharides. *TIBTECH.* 6, 1988.

Nilsson, K.G.I. (1989). Enzymic synthesis of di- and tri-saccharide glycosides, using glycosidases and β-D-galactoside 3-α-sialyl-transferase. *Carbohydrate research.* 188, 9-17.

Onishi, N. and Tanaka, T. (1997). Purification and characterization of galacto-oligosaccharide producing β-galactosidase from *Sirobasidium magnum. Letters in Applied Microbiology.* 24, 82-86.

Ooe, Y., Yamamoto, S., Kobayashi, M. and Kise, H. (1999). Increase of catalytic activity of α-chymotrypsin in organic solvent by co-lyophilization with cyclodextrins. *Biotechnology Letters.* 21, 385-389.

Paulsen, H. (1984). Synthesis of complex oligosaccharides chains of glycoprotein. *Chemical social reviews*. 13, 15-45.

Pelton, J.T. (2001). Secondary Considerations. *Science.* 291(2211), 2175-2176.

Rastall, R.A. (2004). Bacteria in the Gut: friends and foes and how to alter the balance. In Finley, D., Morris, J.G. and Rogers, Q.R. (eds.), *Proceeding of WALTHAM International Science Symposium: Nature, Nurture, and the Case for Nutrition,* pp.2022S-2026S. Bangkok, 2003.

Reuter, S., Nygaard, A.R. and Zimmermann, W. (1999). β-Galactooligosaccharide synthesis with β-galactosidases from *Sulfolobus solfataricus, Aspergillus oryzae,* and *Escherichia coli. Enzyme and Microbial Technology.* 25, 509-516.

Roberfroid, M.B. (1997). Health benefits of non-digestible oligosaccharides. In Kritchevsky, D. and Bonfield, C. (eds.), *Dietary Fiber in Health and Disease.* Plenum Press. New York. pp. 211-219.

Roberfroid, M.B. and Slavin, J. (2000). Nondigestible oligosaccharides. *Critical Reviews in Food Science and Nutrition.* 40, 461-480.

Rudick, M.J. and Elbein, A.D. (1975). Glycoprotein enzymes secreted by *Aspergillus fumigatus*: purification and properties of a second β-glucosidase**.** *Journal of bacteriology.* 124, 534-541.

Sako, T., Matsumoto, K. and Tanaka, R. (1999). Recent progress on research and applications of non-digestible galacto-oligosaccharides. *International Dairy Journal.* 9, 69-80.

Santos, A.M., Clemente, I.M., Barletta, G. and Griebenow, K. (1999). Activation of serine protease subtilisin Carlsberg in organic solvents: Combined effect of methyl-β-cyclodextrin and water. *Biotechnology letters.* 21, 1113-1118.

Sauerbrei, B. and Thiem, J. (1992). Galactosylation and glucosylation by use of β-galactosidase. *Tetrahedron letters*. 33, 201-204.

Schmidt, R.R. (1986). New methods for the synthesis of glycosides and oligosaccharides - Are there alternative to the Koenis-Knorr method? *Angewandte chemie* (Int. Ed. Engl.). 25(3), 213-236.

Segal, I.H. (1976). Biochemical calculations: how to solve mathematical problems in general biochemistry. 2nd edition. John Wiley & Sons. New York. pp. 12-14.

Simon, L.M., László, K., Vértesi, A., Bagi, K. and Szajáni, B. (1998). Stability of hydrolytic enzymes in water-organic solvent systems. *Journal of Molecular Catalysis B: Enzymatic.* 4, 41-45.

Switzer, R.L. and Garrity, L.F. (1999). Experimental biochemistry. 3rd edition. W.H. Freeman and company. New York. pp. 100-103.

Szejtli, J. (1988). Cyclodextrin Technology. Kluwer Academic Publisher. Dordrecht. Cited in Szejtli, J. (1998). Introduction and general overview of cyclodextrin chemistry. *Chemical Reviews.* 98, 1743-1753.

Szejtli, J. (1998). Introduction and general overview of cyclodextrin chemistry. *Chemical Reviews.* 98, 1743-1753.

Szejtli, J. (2004). Past, present, and future of cyclodextrin research. *Pure and Applied Chemistry.* 76, 1825-1845.

Tanaka, Y., Kagamiishi, A., Kiuchi, A. and Horiuchi, T. (1975). Purification and Properties of β-Galactosidase from *Aspergillus oryzae. Journal of Biochemistry.* 77, 241-247.

The joint FAO/WHO expert committee on food additives (JECFA). (2005). Summary of evaluations performed by the Joint FAO/WHO expert committee on food additives**.** [cited 2 February 2007]. Available from http://www.inchem.org/documents/jecfa/ jeceval/jec_465.htm

Triantafyllou, A.O., Wehtje, E., Adlercreutz, P. and Mattiasson, B. (1997). How do additives affect enzyme activity and stability in nonaqueous media? *Biotechnology and Bioengineering*. 54(1), 67-76.

van der Veen, B.A., Joost, C.M., Uitdehaag, B.W.D. and Lubbert, D. (2000). Engineering reaction and production specificity of cyclodextrin glycosyltransferase from *Bacillus circulans strain* 251. *Biochemica et Biophysica Acta: Protein and proteomic.* 1543(2), 336-360.

Van Laere, K.M.J., Hartemink, R., Beldman, G., Pitsin, S., Dijkema, C., Schols, H.A. and Voragen, A.G.J. (1999). Transglycosidase activity of *Bifidobacterium*

adolescentis DSM 20083 α-galactosidase. *Applied microbiology and biotechnology*. 52, 681-688.

Vocadlo, D.J. and Withers, S.G. (2000). Glycosidase-catalysed oligosaccharide synthesis. In Ernst, B., Hart, G.W. and Sinay, P. (eds.). *Carbohydrates in chemistry and biology: Part I Chemistry of saccharides Vol.2 Enzymatic synthesis of glycosides and carbohydrate-receptor interaction*. Wiley. Weinheim. pp. 723-735.

Voragen, A.G.J. (1998). Technological aspects of functional food-related carbohydrates. *Trends in Food Science & Technology*. 9, 328-335.

Wolfgang, Hosel. and Wolfgang, Barz. (1975). β-Glucosidases from *Cicer arietinum* L. purification and properties of isoflavone-7-O-glucoside-specific beta-glucosidases. *European Journal of Biochemistry*. 57, 607-616.

Yoon, J.H. and Mckenzie, D. (2005). A comparison of the activities of three β-galactosidases in aqueous-organic solvent mixtures. *Enzyme and Microbial Technology*. 36, 439-446.

Yu, Y., Li, R., Xu, C., Ruan, K., Shen, Y. and Govindjee. (2001). *N*-bromosuccinimide modification of tryptophan 241 at the C-terminus of the manganese stabilizing protein of plant photosystem 11 influences its structure and function. *Physiologia Plantarum*. 111,108-115.

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APPENDICES

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APPENDICES

Appendix 1: Preparation for SDS-Polyacrylamide gel electrophoresis

1) **Stock reagents**

2 M Tris-HCl pH 8.8

Tris(hydroxymethyl)-aminomethane	24.2	g

Adjusted pH to 8.8 with 1 M HCl and adjusted volume to 100 ml with distilled water

1 M Tris-HCl pH 6.8

Tris(hydroxymethyl)-aminomethane	12.1	g

Adjusted pH to 6.8 with 1 M HCl and adjusted volume to 100 ml with distilled water

10% (w/v) SDS

Sodium dodecyl sulfate	10	g

Adjusted volume to 100 ml with distilled water

50% (v/v) glycerol

100% glycerol	50	ml

Added 50 ml distilled water

1% (w/v) bromophenol blue

Bromophenol blue	100	mg

Brought to 10 ml with distilled water and stir until dissolved. Filtration will remove aggregated dye.

2) **Working Solutions**

Solution A

30% Acrylamide, 0.8% bis-acrylamide, 100 ml

Acrylamide	29.2	g
N,N'-methylene-bis-acrylamide	0.8	g

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Adjusted volume to 100 ml with distilled water

Solution B

4x Seperating Gel Buffer

2 M Tris-HCl pH 8.8	75	ml
10% SDS	4	ml
Distilled water	21	ml

Solution C

4x Stacking Gel Buffer

1 M Tris-HCl pH 6.8	50	ml
10% SDS	4	ml
Distilled water	46	ml

10% ammonium persulfate

Ammonium persulfate	0.5	g
Distilled water	5	ml

Electrophoresis Buffer

Tris(hydroxymethyl)-aminomethane	3	g
Glycine	14.4	g
Sodium dodecyl sulfate	1	g

Adjusted volume to 1 liter with distilled water

5x Sample Buffer

1M Tris-HCl pH 6.8	0.6	ml
50% glycerol	5	ml
10% SDS	2	ml
2-mercaptoethanol	0.5	ml
1% bromophenol blue	1	ml
Distilled water	0.9	ml

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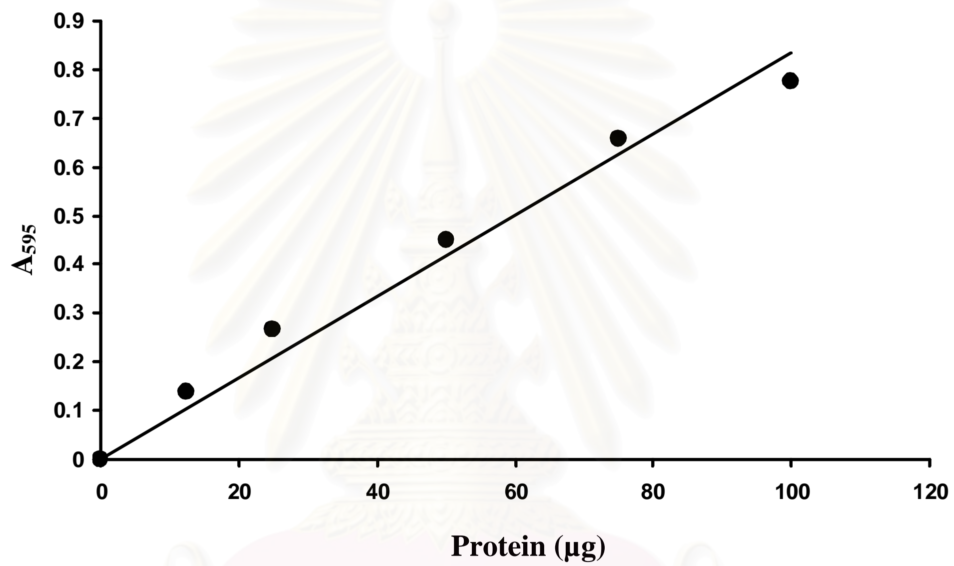
Appendix 2:Standard curve for protein determination by Bradford's method



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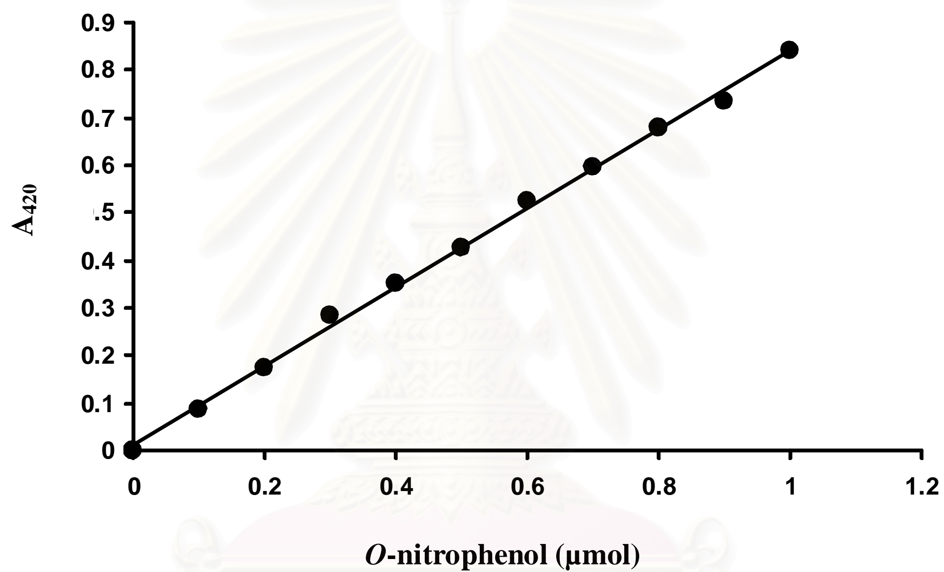
Appendix 3:Standard curve of *O*-nitrophenol by hydrolysis activity assay



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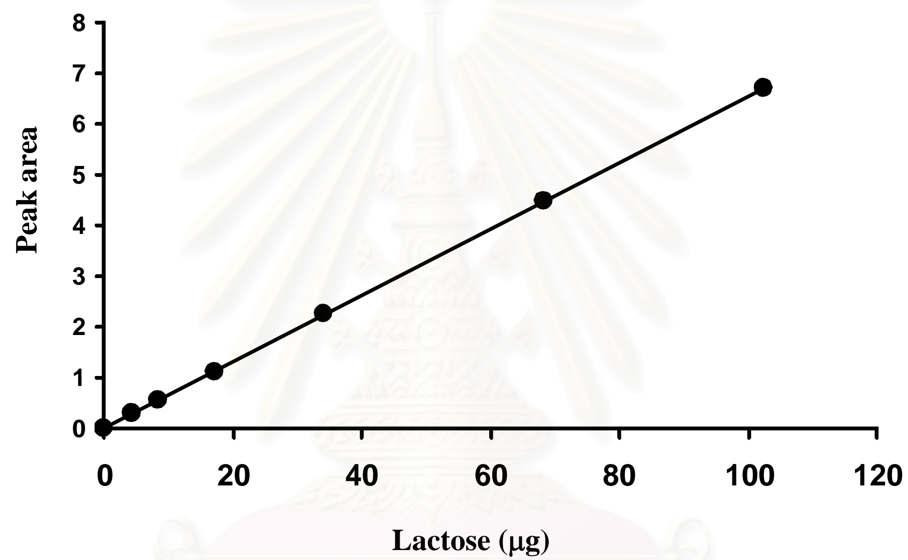
Appendix 4:Standard curve of lactose by thin layer chromatography (TLC)



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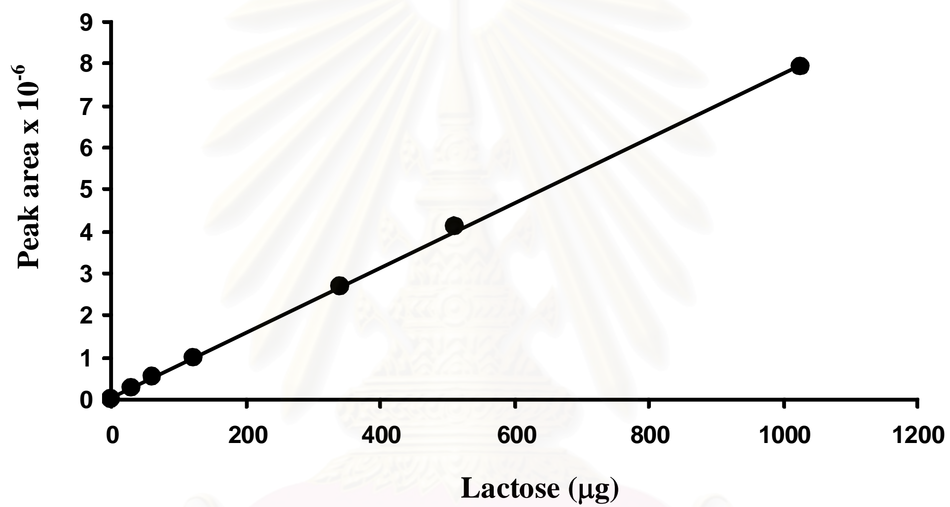
Appendix 5:Standard curve of lactose by high performance liquid chromatography (HPLC)



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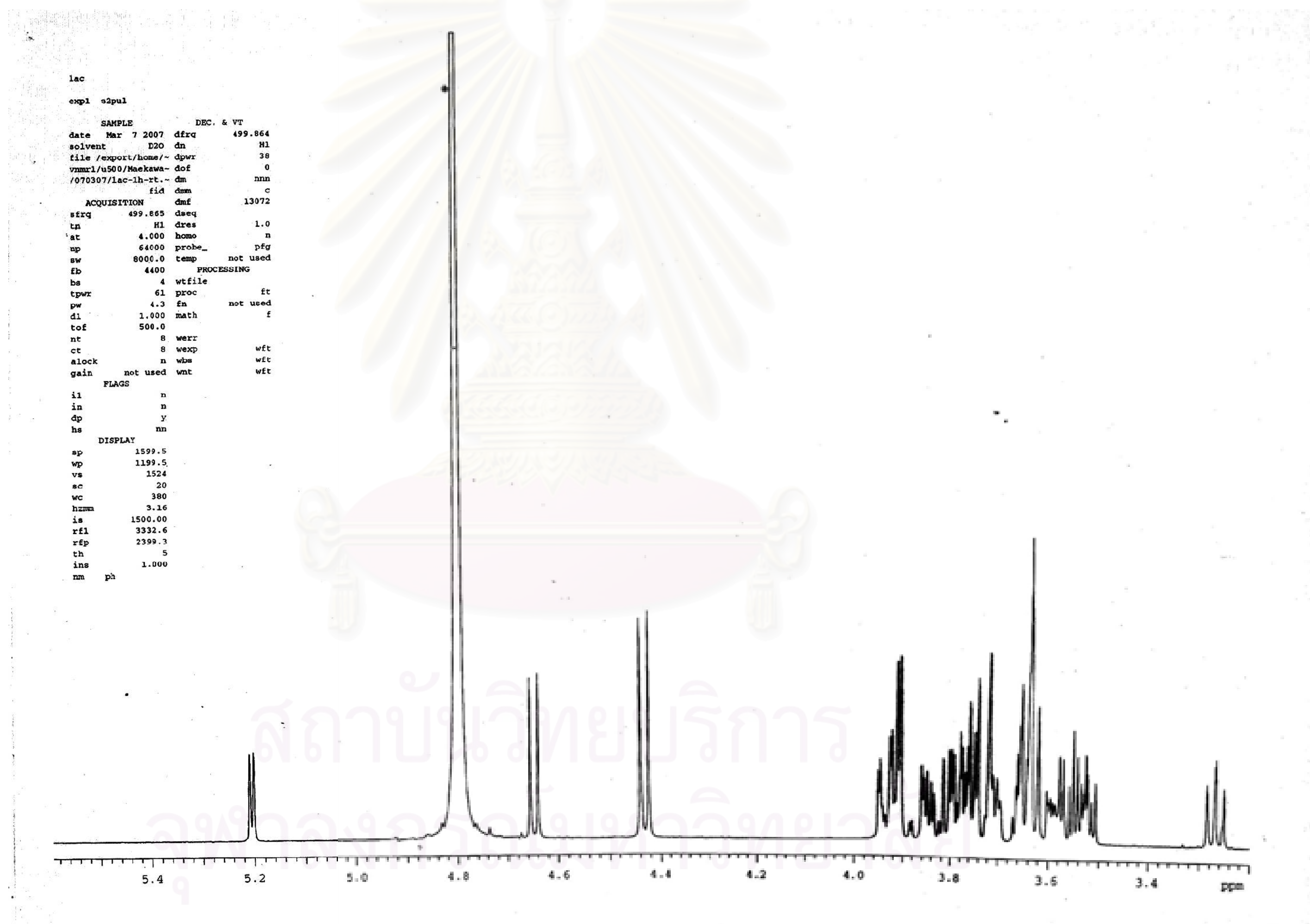
Appendix 6: The 500 MHz ^{1}H-NMR spectra of lactose



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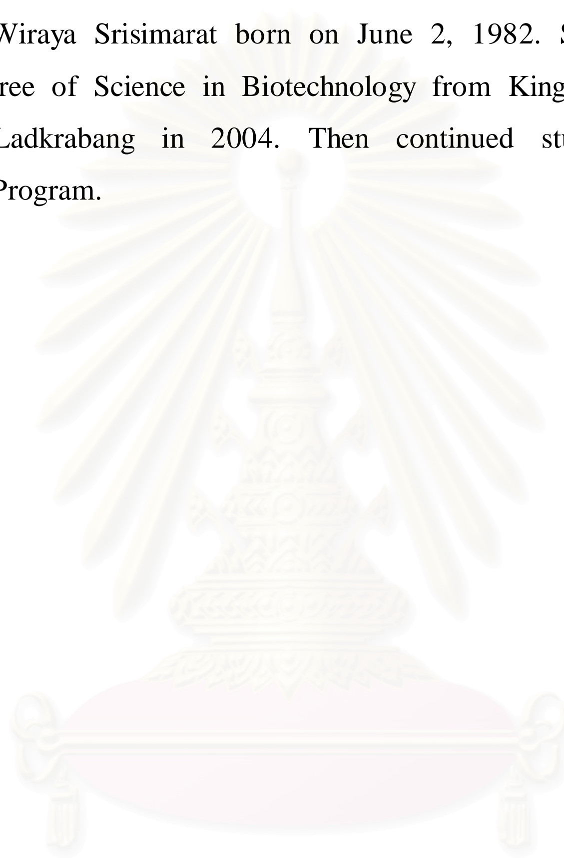
BIOGRAPHY

Miss Wiraya Srisimarat born on June 2, 1982. She graduated with the Bachelor Degree of Science in Biotechnology from King Mongkut's Institute of Technology Ladkrabang in 2004. Then continued studying for Master in Biochemistry Program.



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