ปฏิกิริยาแทรนส์ไกลโคซิเลชันที่เร่งโดยไซโคลเดกซ์ทรินไกลโคซิลแทรนส์เฟอเรสและแอมิโลมอลเทสเพื่อการสังเคราะห์ออลิโกแซ็กคาไรด์ที่มีสมบัติต้านฟันผุ

นางสาวสิริวิภา แซ่หู

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2553

TRANSGLYCOSYLATION REACTION CATALYZED BY CYCLODEXTRIN GLYCOSYLTRANSFERASE AND AMYLOMALTASE FOR THE SYNTHESIS OF OLIGOSACCHARIDES WITH ANTICARIOGENIC PROPERTY

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science program in Biochemistry
Department of Biochemistry
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Thesis Title	TRANSGLYCOSYLATION REACTION CATALYZED BY CYCLODEXTRIN GLYCOSYLTRANSFERASE AND AMYLOMALTASE FOR THE SYNTHESIS OF OLIGOSACCHARIDES WITH ANTICARIOGENIC PROPERTY
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(Associate Professor Rudee Surarit, Ph. D.)

สิริวิภา แซ่หู : ปฏิกิริยาแทรนส์ไกลโคซิเลชันที่เร่งโดยไซโคลเดกซ์ทรินไกลโคซิลแทรนส์เฟอเรสและแอมิโลมอลเทสเพื่อการสังเคราะห์ออลิโกแซ็กคาไรด์ที่มีสมบัติต้านฟันผุ. (TRANSGLYCOSYLATION REACTION CATALYZED BY CYCLODEXTRIN GLYCOSYLTRANSFERASE AND AMYLOMALTASE FOR THE SYNTHESIS OF OLIGOSACCHARIDES WITH ANTICARIOGENIC PROPERTY) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ดร. เปี่ยมสุข พงษ์สวัสดิ์, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร.มัญชุมาส เพราะสุนทร, 112 หน้า.

งานวิจัยนี้ได้ศึกษาการสังเคราะห์มอลโทซิลซูโครสโดยใช้ไกลโคซิลแทรนส์เฟอเรส (CGTase) จาก *Paenibacillus* sp.RB01 และรีคอมบิแนนท์แอมิโลมอลเทส จาก *Corynebacterium glutamicum* ในการโยกย้ายหมู่กลูโคซิลจากตัวให้หลายชนิดไปยังตัวรับซูโครส งานนี้เป็นงานวิจัยแรกที่รายงานการใช้แอมิโลมอลเทสเพื่อสังเคราะห์มอลโทซิลซูโครส ในการวิเคราะห์ผลิตภัณฑ์ที่ได้ด้วยเทคนิค TLC และ HPLC พบว่าตัวให้หมู่กลูโคซิลที่เหมาะสมสำหรับ CGTase และ แอมิโลมอลเทส คือ แป้งมันสำปะหลังที่ละลายน้ำได้และแป้งมันสำปะหลังดิบ ภาวะที่เหมาะสมเพื่อให้ได้ผลผลิตสูง คือ บ่มซูโครส 2.5%(w/v), แป้งมันสำปะหลังที่ละลายน้ำได้ 20% (w/v) กับ CGTase 400 U/ml เป็นเวลา 18 ชั่วโมง หรือบ่มซูโครส 2.0%(w/v), แป้งมันสำปะหลังดิบ 2.5%(w/v) กับแอมิโลมอลเทส 9 U/ml เป็นเวลา 48 ชั่วโมง โดยพบว่า CGTase ให้ผลิตภัณฑ์มากกว่าแอมิโลมอลเทส ปริมาณผลิตภัณฑ์ที่ได้เมื่อคิดเทียบกับปริมาณน้ำตาลซูโครสเริ่มต้น คือ 98% และ 81% ตามลำดับ จากนั้นทำการแยกผลิตภัณฑ์ด้วยคอลัมน์ Biogel P-2 แล้วทำการวิเคราะห์แต่ละพีค ด้วยเทคนิค HPLC และ Mass Spectrometry พบว่าผลิตภัณฑ์หลักที่ได้ของทั้งสองเอนไซม์ คือ G_2F, G_3F, G_4F และ G_5F โดยมีอัตราส่วนของผลิตภัณฑ์ที่ต่างกัน คือ 1.0:1.2:1.2:1.3 และ 1.0:0.9:0.8:0.7 จาก CGTase และ แอมิโลมอลเทส ตามลำดับ เมื่อใช้ซูโครสและเอนไซม์ในปริมาณน้อยลง พบว่าผลิตภัณฑ์ขนาดใหญ่ (G_7F, G_8F และ $> G_8F$) มีอัตราส่วนเพิ่มขึ้น จากนั้นตรวจสอบสมบัติการต้านฟันผุ โดยวัดการสังเคราะห์กลูแคนที่ไม่ละลายน้ำ การผลิตกรด การก่อให้เกิดคราบพลักซ์ และ การรวมกลุ่มของเซลล์ ของ *Streptococcus mutans* พบว่ามอลโทซิลซูโครสมีสมบัติที่ก่อให้เกิดฟันผุได้ต่ำกว่าเมื่อเปรียบเทียบกับน้ำตาลซูโครส และเมื่อนำไปศึกษาการยับยั้งแอกทิวิตีของ กลูโคซิลแทรนส์เฟอเรส (GTase) พบว่าในสารละลายซูโครสผสมมอลโทซิลซูโครสในอัตราส่วน 1:1, 1:2 และ 1:4 พบว่า สามารถยับยั้งแอกทิวิตีของ GTase ได้ 7, 33 และ 50% ตามลำดับ จากผลการทดลองนี้ทำให้ทราบว่ามอลโทซิลซูโครสเป็นน้ำตาลที่มีคุณสมบัติก่อให้เกิดฟันผุได้ต่ำ

ภาควิชา.....ชีวเคมี..... ลายมือชื่อนิสิต.....

สาขาวิชา.....ชีวเคมี ..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก……

ปีการศึกษา.....2553 ..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม……...

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KEYWORDS : CYCLODEXTRIN GLYCOSYLTRANSFERASE / AMYLOMALTASE / TRANGLYCOSYLATION / MALTOSYLSUCROSE

SIRIWIPA SAEHU : TRANSGLYCOSYLATION REACTION CATALYZED BY CYCLODEXTRIN GLYCOSYLTRANSFERASE AND AMYLOMALTASE FOR THE SYNTHESIS OF OLIGOSACCHARIDES WITH ANTICARIOGENIC PROPERTY. ADVISOR : PROF. PIAMSOOK PONGSAWASDI, Ph.D., CO-ADVISOR : ASST. PROF. MANCHUMAS PROUSOONTORN, Ph.D., 112 pp.

This work aims to synthesize maltosylsucrose using cyclodextrin glycosyltransferase (CGTase) from *Paenibacillus* sp. RB01 and recombinant amylomaltase from *Corynebacterium glutamicum* through the transglycosylation reaction with various glucosyl donors and sucrose acceptor. This is the first report on using amylomaltase for the synthesis of maltosylsucrose. In the analysis of product by TLC and HPLC on a Rezex RSO-Oligosaccharide column, the suitable glucosyl donor for CGTase and amylomaltase were soluble and raw tapioca starch, respectively. The optimal condition for the synthesis of maltosylsucrose from CGTase was 2.5% (w/v) sucrose, 20% (w/v) tapioca starch (soluble), 400 U/mL (dextrinizing unit) of CGTase at 40°C for 18 hours and that for amylomaltase was 2.0% (w/v) sucrose, 2.5% (w/v) tapioca starch (raw), 9 U/ml (disproportionation unit) of amylomaltase at 30°C for 48 hours. CGTase was more efficient than amylomaltase for this synthetic reaction in which the total percent yields of the transfer products from CGTase and amylomaltase were about 98% and 81%, respectively. The products were isolated by Biogel P-2 gel column, each peak was analyzed by HPLC and mass spectrometry. The major transfer products of both enzymes were G_2F, G_3F, G_4F and G_5F with different product ratio. The ratios were 1.0:1.2:1.2:1.3 and 1.0:0.9:0.8:0.7 for CGTase and amylomaltase, respectively. Higher ratio of larger products (G_7F, G_8F and > G_8F) could be obtained from both enzymes at low amounts of sucrose and enzyme. Low cariogenic property of maltosylsucrose products was confirmed by comparing the synthesis of water insoluble glucan, acid fermentation, plaque formation and cell aggregation of *Streptococcus mutans* to those exerted by sucrose. By adding sucrose to maltosylsucrose products in the ratios of 1:1, 1:2 and 1:4, inhibitory effects on glucosyltransferase activity of *S. mutans* by 7, 33 and 50% were observed. These results suggested that maltosylsucrose products were low cariogenic oligosaccharides.

Department : Biochemistry Student's Signature .....

Field of Study : Biochemistry Advisor's Signature .....

Academic Year : 2010 Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

A	Absorbance
BSA	Bovine serum albumin
CDs	Cyclodextrins
CGTase	Cyclodextrin glycosyltransferase
cm	Centimeter
°C	Degree Celsius
Da	Dalton
DP	Degree of Polymerization
ESI-TOF-MS	Electrospray Ionization-Time of Flight Mass Spectrometry
et al.	Et. Alii (latin), and others
g	Gram
h	Hour (s)
HPLC	High Performance Liquid Chromatography
kDa	kiloDalton
L	Litre
µg	Microgram
µl	Microlitre
M	Molar
Min	minute
mg	Milligram
ml	Millilitre
MW	Molecular weight

NDOs	Non digestible oligosaccharides
PAGE	Polyacrylamide gel electrophoresis
Rf	Relative mobility
Rt	Retention time
SDS	Sodium dodecyl sulfate
U	Unit(s)
v/v	Volume by volume
w/v	Weight by volume
WIG	Water insoluble glucan

CHAPTER I

INTRODUCTION

1.1 Anticariogenic oligosaccharide

1.1.1 The cause of dental caries

Dental caries is one of the most common oral diseases that caused by three major factors, i.e., teeth, oral bacteria, and dietary carbohydrates (Hamada, 2002). The oral bacteria including *Streptococcus mutans*, is main causative of dental caries that produces a water-insoluble glucan from sucrose via the activity of glycosyltransferase. The water-insoluble glucan covers the dental surface, forming a caries-conductive plaque and attachment of the bacterial cells. In the presence of a saccharide such as glucose, fructose or sucrose, bacterial acid production in the plaque occurs which subsequently initiates the occurrence of dental caries (Otaka, 2006) (Figure 1.1).

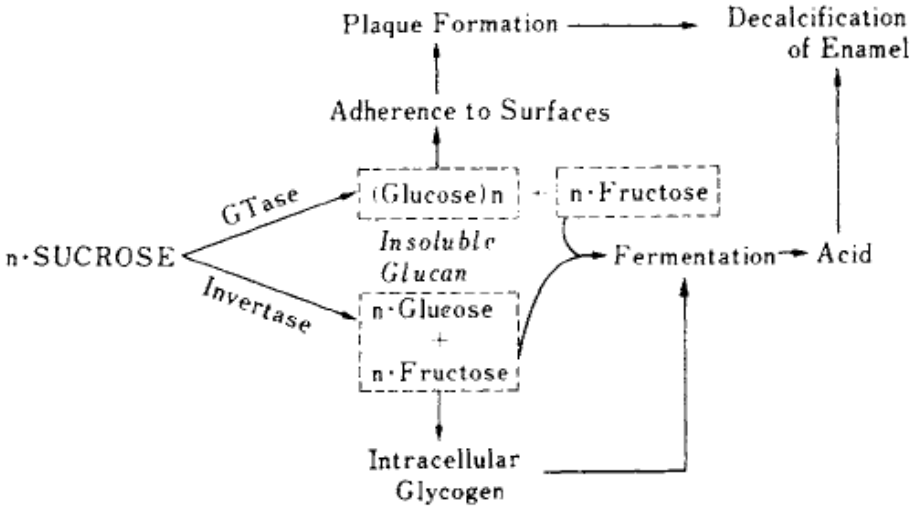


Figure 1.1 Utilization of sucrose by *Streptococcus mutans* and pathogenesis of dental caries (Hamada *et al*., 1984)

The dental plague formation by adherence of S. mutans involves two steps: an initial step was a reversible interaction between the bacterial and tooth surfaces with glycoprotein as binding site. The second step was an irreversible reaction in which the microbes synthesize a water-insoluble glucan from sucrose and free fructose is released by the action of a Glucosyltransferase (GTase), an extracellular enzyme that transfers glucose from sucrose to produce a polymer glucan (Hamada *et al*., 1984). Furthermore, GTase is able to induce the aggregation of cells to dental plaque which is performed for lactic acid production from fermentable fructose and other sugars present in daily foods. When the pH at the tooth surface was below 5.5, decalcification of enamel or dentin of the tooth occurs (Decker and van Loveren, 2003). Sucrose substitutes are not function as substrate and not utilized by *S. mutans* for the synthesis of insoluble glucan. Therefore, acid production by dental plaque suspensions is lower in the presence of anticariogenic oligosaccharides compared with sucrose. Furthermore, they have been shown to have reduced cariogenic potential compared with sucrose.

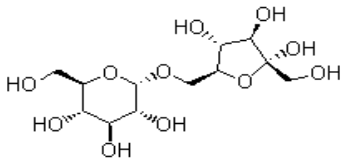
1.1.2 Prevention of dental caries

Dental caries can be prevented by using fluoride as caries-limiting agents that facilitates remineralization of enamel. The second approach is to use sucrose substitutes which interrupt the process of the adherence of S. mutans to the tooth surface.

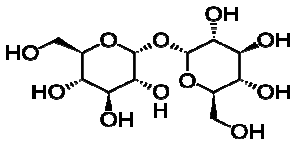
Sucrose substitutes are food additive that give food a sweet taste, are currently available as ingredients of a variety of candy, chewing gum, and drinks. They are classified as carbohydrate sweeteners (caloric) and non-carbohydrate sweeteners (non-caloric). Carbohydrate sweeteners include various oligosaccharides, starch sugars and sugar alcohol and non-carbohydrate sweeteners are divided into chemically synthesised sweeteners, including saccharin, aspartame and sucralose, and those obtained from plants, such as stevioside, thaumatins and monellin (Matsukubo

and Takazoe, 2006). The structural formulae of examples of sucrose substitutes are shown in Figure 1.2.

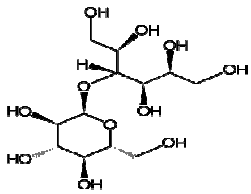
Furthermore, many anticariogenic oligosaccharides have been developed and commercially produced as bulking sucrose substitutes. They have beneficial additional health effects such as offering lower available energy, saving insulin secretion from the pancreas, improvement of intestinal microflora, stimulation of intestinal mineral absorption and prevention of cancer (Oku and Nakamura, 2002).



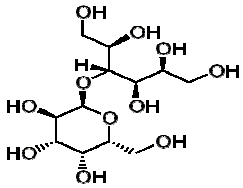
Palatinose
α-D-glucopyranosyl-1, 6-D-fructose



Trehalose
α-D-glucopyranosyl-1, 1-D-glucose



Maltitol
4-O-α-D-Glucopyranosyl-D-glucitol



Lactitol
4-O-α-D-Galactopyranosyl-D-glucitol

Figure 1.2 The structural formulae of sucrose substitutes ((Matsukubo and Takazoe, 2006)

1.1.3 Properties of oligosaccharides

The carbohydrates can be classified according to their molecular size or degree of polymerization (number of monosaccharide units combined), into monosaccharides, oligosaccharides and polysaccharides. Oligosaccharides are defined as saccharides containing between 3 and 10 sugar moieties. Other authorities classify saccharides including anyone from 3 to 19 monosaccharide units in this group (Mussatto and Mancilha, 2007). Oligosaccharides have been utilized in food production with interesting funtional properties and they are water soluble and mildly sweet, typically

0.3-0.6 times as sweet of sucrose which depend on chemical structure in the degree of polymerization and the levels of mono- and disaccharide in the mixture (Crittenden and Playne, 1996).

Several properties of oligosaccharides are shown in Table 1.1. The interesting properties of oligosaccharides are the relatively low sweetness which makes the oligosaccharides useful in food production when a reduced sweetness is desirable to enhance other food flavors, and the ability to alter the freezing temperature of frozen foods. In addition, oligosaccharides help to control the intensity of browning due to Maillard reactions in heat-processed foods and 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 1.1 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 enzymes, enzyme inhibitors, elicitors, etc.

1.1.4 The health benefits of oligosaccharides

A dietary oligosaccharide that is not digested and absorbed in the small intestine, namely non-digestible oligosaccharides (NDOs), reaches the large intestine where it is completely fermented by intestinal bacteria and produces short-chain fatty acids, which are converted to energy. The pathway of energy production from NDOs is shown in Figure 1.3.

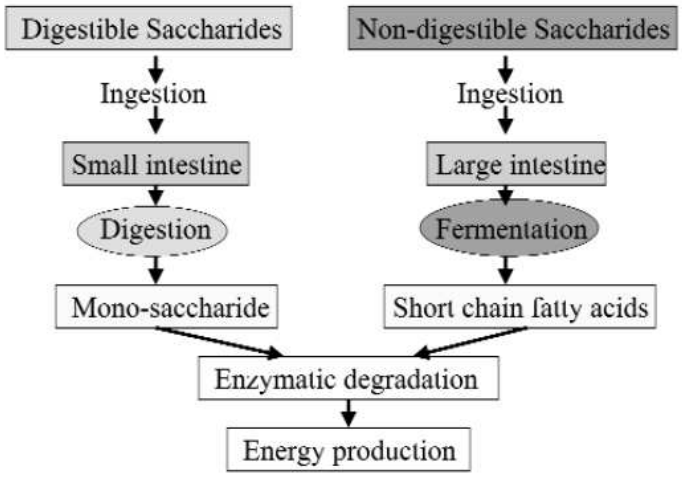


Figure 1.3 Pathway of energy production from nondigestible sucrose substitute (Oku and Nakamura, 2002)

NDOs is not digested by gastrointestinal digestive enzymes as either results from the configuration of the glycosidic bond between monomeric sugar units or the substrate selectivity. Most NDOs have a *β*-configuration and cannot be degraded by human gastrointestinal digestive enzymes, which are specific for *α*-glycosidic bonds. Such compounds include carbohydrates where fructose, galactose, glucose and/or xylose are the monosaccharides unit present. However, there are NDOs with *α*-configuration and which in principle can be degraded by human *α*-glycolytic enzymes, but the degradation is at low level because the enzymes usually have only weak activities and/or show other substrate selectivity (Swennen *et al*., 2006). This property makes the NDOs suitable for use in sweet, low-caloric diet foods, and for consumption by individuals with diabetes (Mussatto and Mancilha, 2007). The NDOs are known to promote the growth of probiotics (bacteria in the large intestine) mainly the *Bifidobacteria* species, and thus recognized as prebiotics. Commercially produced NDOs are grouped into 13 classes (Table 1.1).

Indigestibility of NDOs gives rise to fermentation in the large intestine, these 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, known as fermentation, not only serves the bacteria by providing energy for proliferation, but 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, NDOs are indirect energy substrates and metabolic regulators (Delzenne and Roberfroid, 1994). The amounts and types of SCFA produced in the colon depend on the type of NDO substrate as well as on the composition of the intestinal flora (Sako *et al*., 1999). NDOs thus give rise to an increase of *Bifidobacteria* and SCFA production. The indigestibility leads to diversify physiological functions, and the functions are classified into three types as shown in Fig. 1.4 (Hirayama, 2002).

Table 1.2 Non-digestible oligosaccharides with bifidogenic functions commercially available (Sako *et al*., 1999)

Compound	Molecular structure*
Cyclodextrins	$(Gu)_n$
Fructooligosaccharides	$(Fr)_n$–Gu
Galactooligosaccharides	$(Ga)_n$–Gu
Gentiooligosaccharides	$(Gu)_n$
Glycosylsucrose	$(Gu)_n$–Fr
Isomaltooligosaccharides	$(Gu)_n$
Isomaltulose (or palatinose)	$(Gu–Fr)_n$
Lactosucrose	Ga–Gu–Fr
Lactulose	Ga–Fr
Maltooligosaccharides	$(Gu)_n$
Raffinose	Ga–Gu–Fr
Soybean oligosaccharides	$(Ga)_n$–Gu–Fr
Xylooligosaccharides	$(Xy)_n$

* Ga, Galactose; Gu, Glucose; Fr, Fructose; Xy, Xylose

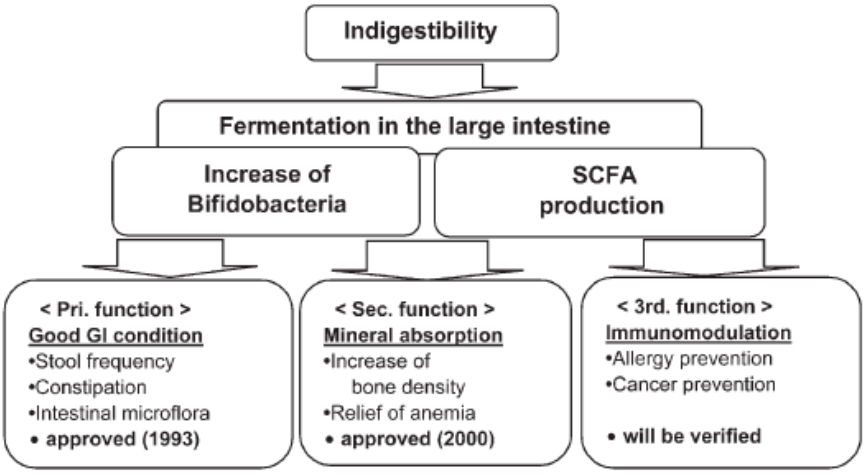


Figure 1.4 An overview of physiological functions of NDOs and their key properties (Hirayama, 2002)

1.2 Production of oligosaccharides

Oligosaccharides can be produced by three methods, they are either extracted from natural sources, obtained by enzyme processing, or produced chemically. At present, enzymatic production of oligosaccharide is the most preferred method due to high specificity, high yield, mild condition and less steps.

1.2.1 Extraction from natural sources

Oligosaccharides can be extracted from different sources. The most prominent example are fructans (e.g. inulin) which are common to edible parts of a variety of plants such as Jerusalem artichoke, chicory root, dahlia tubers, garlic, asparagus root, and salsify. Soybean oligosaccharide, raffinose and stachyose are commercially produced by direct extraction from soybean whey (Swennen *et al*., 2006). However, this method requires natural sources that contain large amount of oligosaccharides, and extraction process may be of high cost.

1.2.2 Chemical production

The chemical synthesis of oligosaccharides in general involves the linking of at least two compounds with multiple hydroxyl groups by the formation of glycosidic

bonds. This bond is formed by a nucleophilic displacement of a leaving group (X) attached to the anomeric carbon of a sugar moiety by an alkoxy group from an alcohol ROH or a partially protected sugar moiety (Figure 1.5) (Weijers *et al*., 2008).

The synthesis of oligosaccharides comprises of the challenge of stereospecifically assembling large highly functionalized complex molecules in an efficient manner, thus chemical methods for the synthesis are complicated by the many protection steps that are necessary for regioselective synthesis (Figure 1.6) (Varki *et al*., 1999). In addition, the number of synthetic steps increases with the size of oligosaccharides that total yields are often low and large-scale synthesis is not practical. It is important that stereospecific reactions are maintained to produce the correct anomer of the target oligosaccharide (Nilsson, 1988).

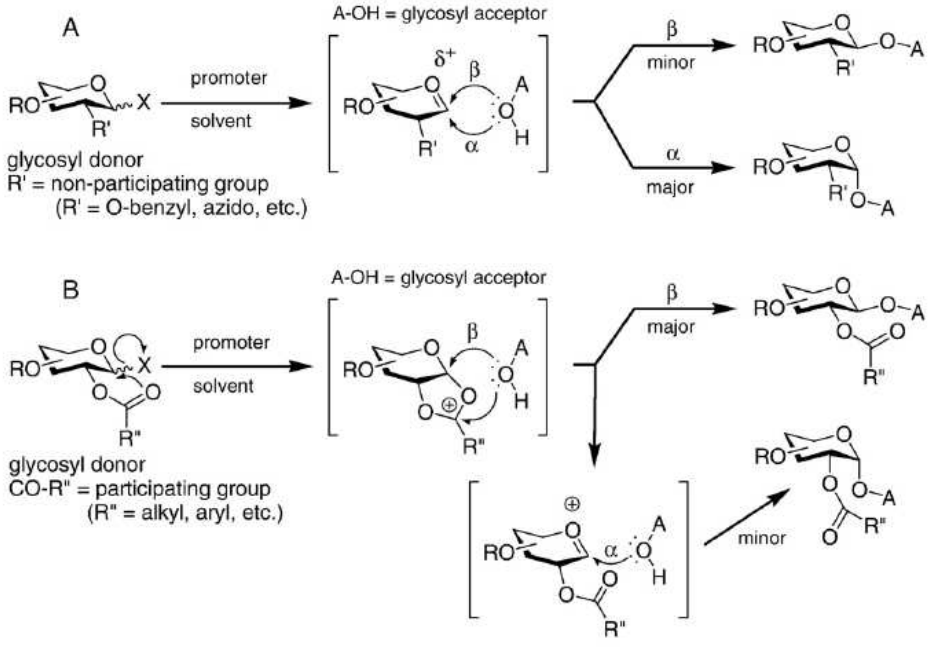


Figure 1.5 General mechanisms for glycosidic bond formation (Weijers *et al*., 2008)

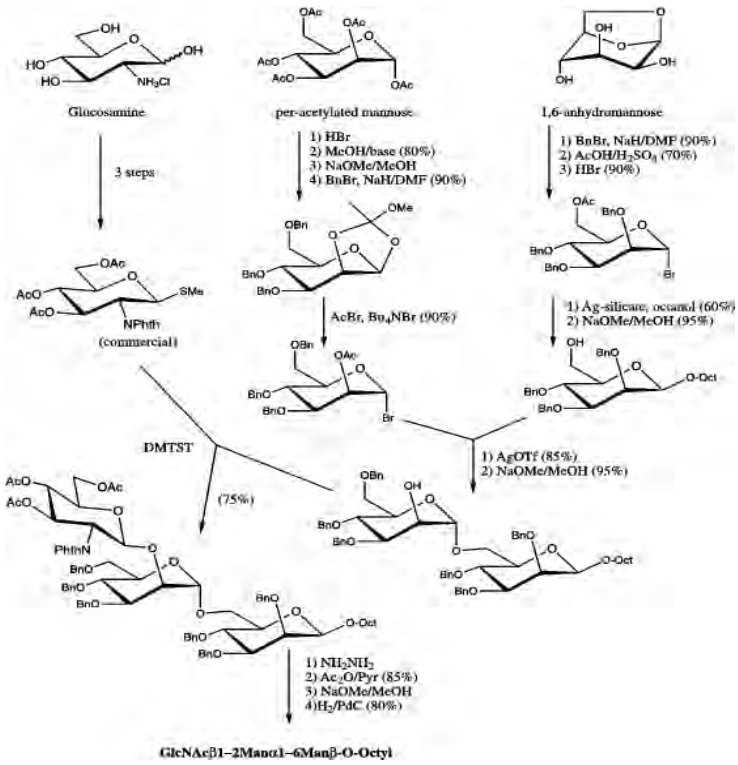


Figure 1.6 Chemical synthetic scheme used for the preparation of a trisaccharide (Varki *et al*., 1999)

1.2.3 Enzyme processing

The very high current level of interest in using enzymes to synthesize oligosaccharides on a preparative scale is in direct response to the severe limitations still present in the chemical approach. The attractiveness of enzymatic synthesis is that protecting groups are not required and that stereochemically defined glycosidic linkages, not racemic α/β mixtures, are always formed. These enzymatic reactions are taken place under mild condition by a simple one-step reaction in which organic solvents and hazardous chemicals or catalysts can be avoided. It is obvious that enzymatic synthesis of oligosaccharides is far more efficient than chemical synthesis of the same structure. Both glycosyltransferases (the enzymes that biosynthesize the oligosaccharides) and glycosidases (the enzymes that hydrolyze them) have been used for synthesis of oligosaccharides (Varki *et al*., 1999).

1.2.3.1 Glycosyltransferase

Glycosyltransferases (EC 2.4) catalyze the transfer of a sugar moiety from an activated donor sugar onto specific saccharide and nonsaccharide acceptors,

to growing oligosaccharide chains. Two main groups of glycosyltransferases can be distinguished: those belonging to the Leloir and non-Leloir enzymes. Glycosyltransferases that use a nucleoside mono- or diphosphate (CMP, UDP, GDP or TDP) to form a sugar nucleotide are called Leloir glycosyltransferase, and non-Leloir glycosyltransferases that utilize sugar (mono) phosphate, or non-activated in the form of sucrose or starch-derived oligosaccharides (Figure 1.7) (Weijers *et al*., 2008). Some non-Leloir glycosyltransferases have been utilized for industrial oligosaccharide synthesis such as fructotransferase and cyclodextrin glycosyltransferase.

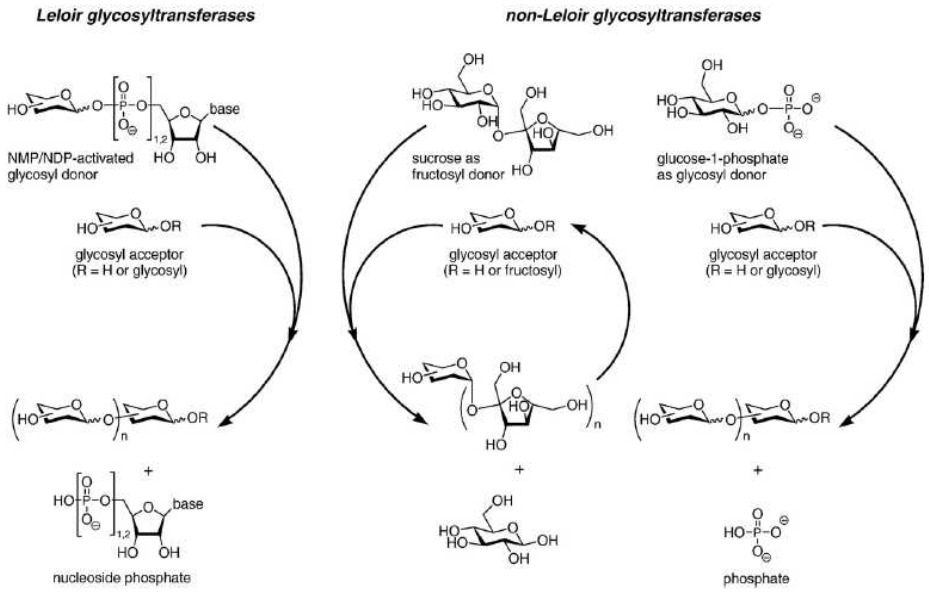


Figure 1.7 Enzymatic synthesis of oligosaccharides by Leloir glycosyltransferases, and of oligofructosides and oligosaccharides by non-Leloir glycosyltransferases (Weijers *et al*., 2008).

1.2.3.2 Glycosidase

Glycosidases (EC 3.2.1), also called glycosyl hydrolases, are a group of enzymes responsible for the cleavage of glycosidic linkages. The glycosidase is divided into two groups: exo-glycosidases that cleave glycosidic bonds from the non-reducing end of the oligosaccharide and can invert (α→β, β→α) the stereochemical

configuration at the anomeric carbon of the reducing end (i.e β-amylase and glucoamylase) (Figure 1.8 A). For endo-glycosidases, these enzymes that cleave internal glycosidic bond and the stereochemistry of the anomeric carbon is retained (α→α, β→β) (Figure 1.8 B) (Nilsson, 1988; Weijers *et al.*, 2008).

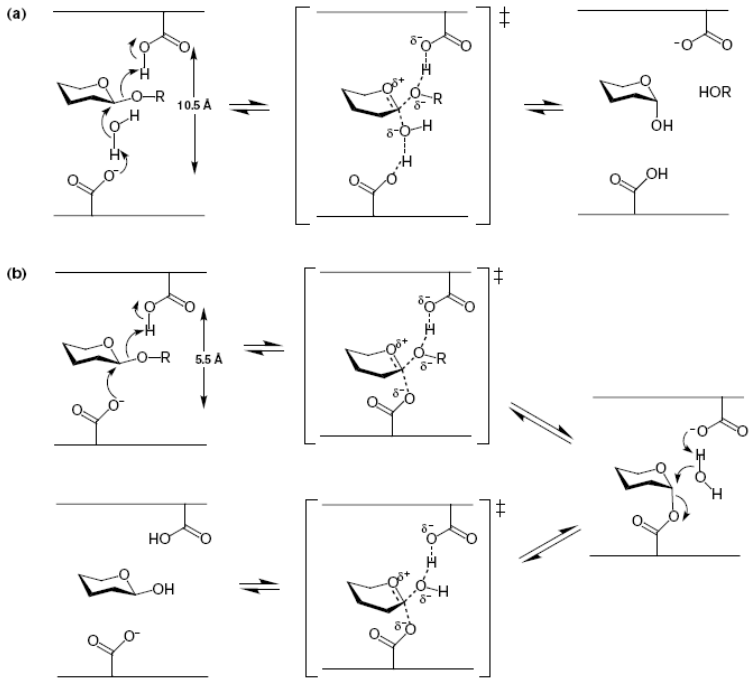


Figure 1.8 General glycosidase mechanisms for (a) an inverting β-glycosidase and (b) a retaining β-glycosidase proceeding through an intermediate with a $^4C_1$ conformation (Rye and Withers, 2000)

1.3 Cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19)

CGTase is a starch degrading enzyme belonging to the important α-amylase family (family 13) of glycosyl hydrolases . All bacterial CGTases convert starch into a mixture of cyclodextrins (CDs) which are mostly consisted of 6, 7 or 8 α (1, 4)-linked glucose residues (α-, β- or γ-cyclodextrins, respectively) via intramolecular transglycosylation reaction (cyclization). These CDs are cyclic oligosaccharides with a hydrophilic outside and a hydrophobic cavity that enables them to form specific inclusion complexes with small hydrophobic guest molecules. CDs are increasingly used in several industrial and research applications (van der Veen *et al.*, 2000) (Figure 1.9).

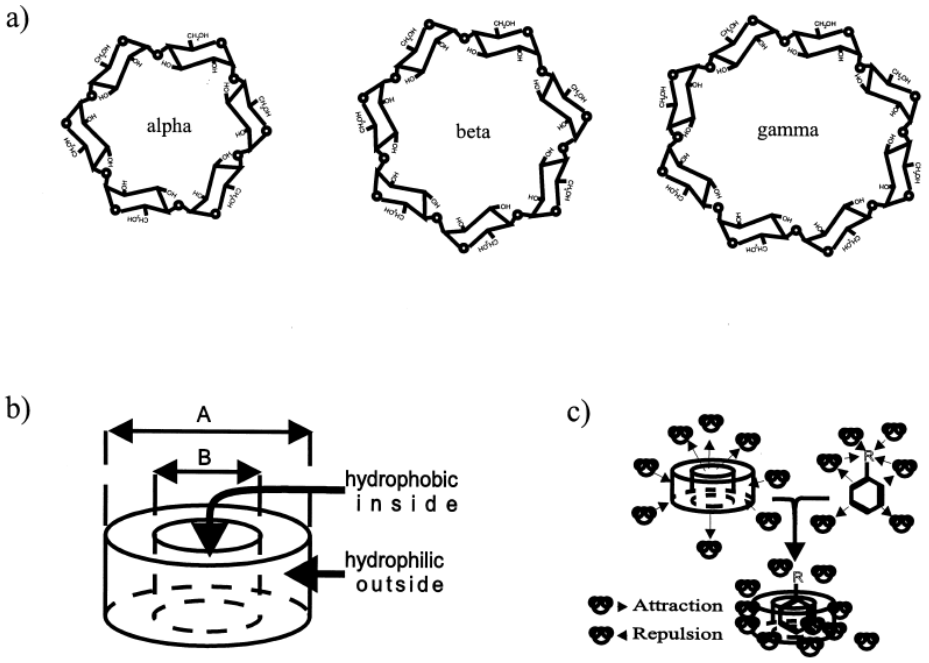


Figure 1.9 Structure and properties of cyclodextrins. (a) α-, β-, and γ-cyclodextrins; (b) the mimical structure and properties of cyclodextrins ; (c) formation of inclusion complex of a cyclodextrin with a hydrophobic guest molecule (van der Veen, 2000)

Besides this cyclization process, CGTases catalyze three other different reactions. Coupling is the reaction in which the CDs molecule is cleaved and transferred to a linear oligosaccharide acceptor to produce a longer linear oligosaccharide. Disproportionation is the major transferase reaction, in which a linear oligosaccharide is cleaved and transferred to another linear oligosaccharide acceptor. And hydrolysis of starch where the glucosyl group is transferred to water acceptor, this activity is low for CGTase (Figure 1.10) (Plou *et al*., 2002; van der Veen *et al*., 2000).

The transglycosylation to saccharides containing a nonreducing-end glucose residue occurs mainly or exclusively at the C-4 hydroxyl group of this glucose moiety (Figure 1.11) (Nakamura *et al*., 1994). It is now well-documented that CGTase has a higher affinity to disaccharides compared with monosaccharides**.** Disaccharides such as isomaltose, gentiobiose, turanose, maltulose, isomaltulose, cellobiose, and sucrose act as good CGTase acceptors (Plou *et al*., 2002).

Moreover, the intermolecular transglycosylation activity of CGTase has been used to synthesize glycosides of several acceptors such as stevioside (Abelyan *et al*., 2004),

dopamine (Yoon *et al*., 2009) and alkyl maltosides (Zhau *et al*., 2008), thus modified properties of the transfer products.

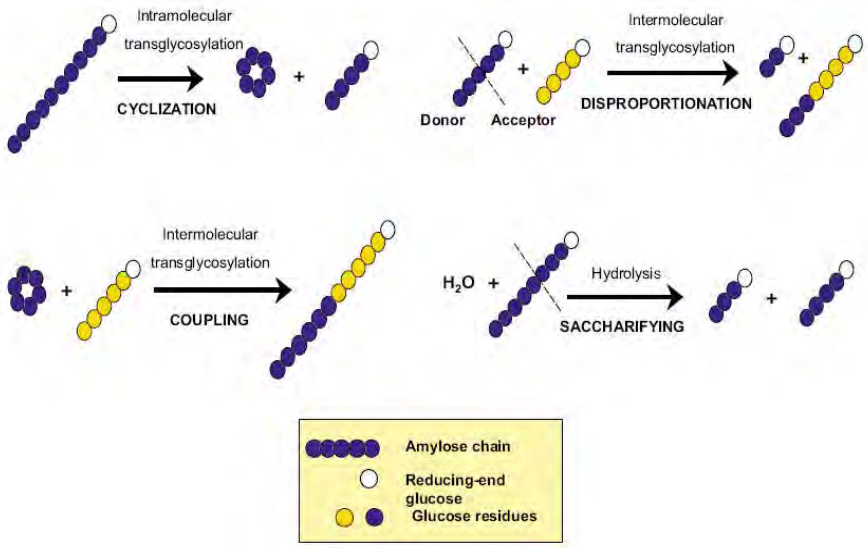


Figure 1.10 Reactions catalyzed by CGTase (Plou *et al*., 2002)

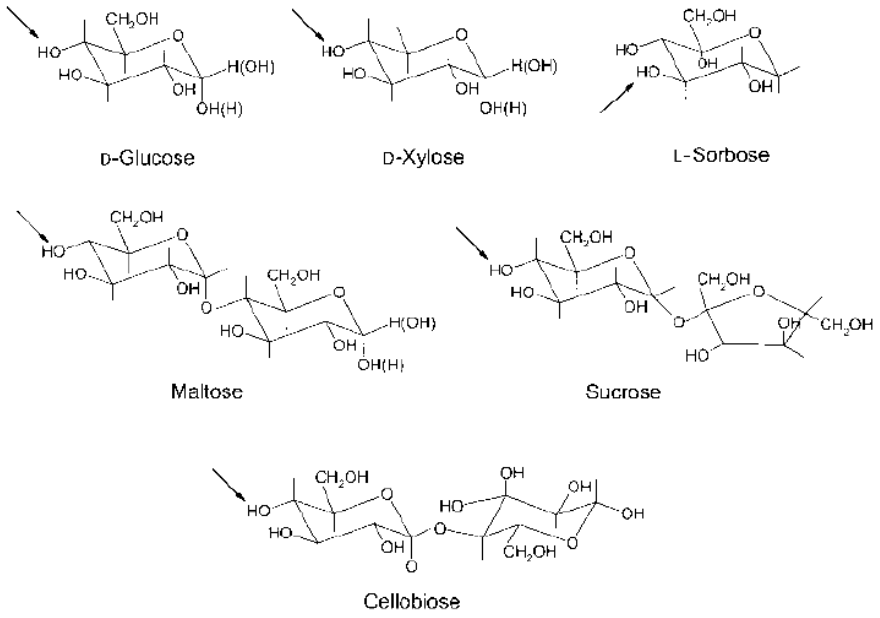


Figure 1.11 Carbohydrates with good acceptor properties for CGTase. The arrows indicate the hydroxyl group where the glucose moiety of the donor is attached (Plou *et al*., 2002)

1.4 Amylomaltase (EC. 2.4.1.25)

Amylomaltase, a member of α-1,4-glycosyltransferase family, is an intracellular enzyme which catalyzes the transglycosylation reaction of α-1,4-D-glucan. It shows a close similarity with CGTase in the catalytic actions, the transfer of glucosyl units via an intermolecular and intramolecular transglucosylation reaction. A unique feature of amylomaltase is its ability to catalyze the formation of large cycloamyloses. In contrast to the well studied cyclodextrin glycosyltransferase (CGTase) which synthesizes cycloamyloses with a ring size (degree of polymerization or DP) of 6-8, amylomaltase is able to produce cycloamyloses or large-ring CDs (LR-CDs) with a degree of polymerization higher than 16 (Figure 1.12) **(**Przylas *et al*., 2000).

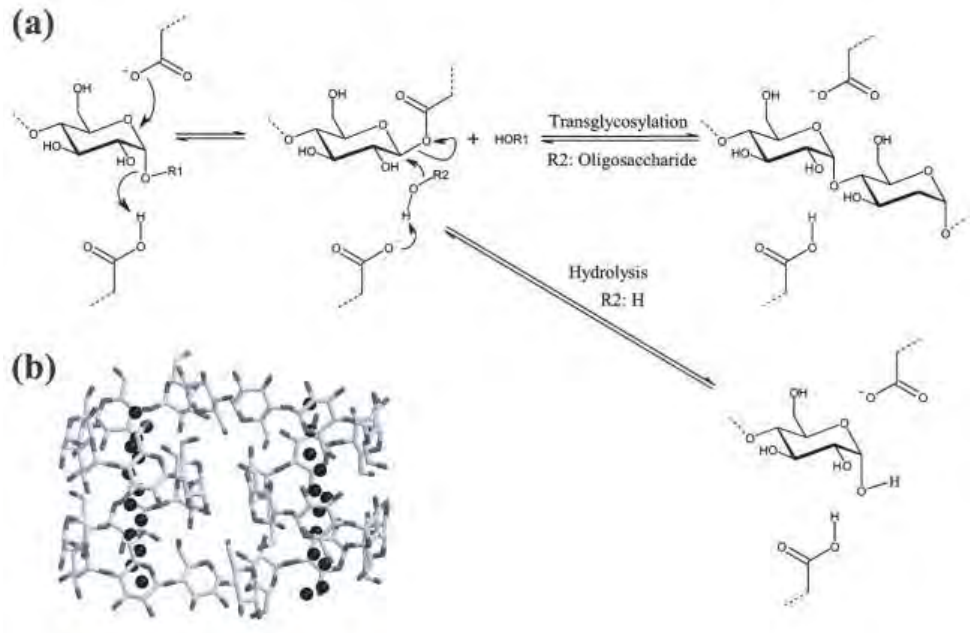


Figure 1.12 Amylomaltase activity (a) Scheme for the reaction pathway of the hydrolysis and transglycosylation activity of amylomaltase (b) Structure of CA-26, a cycloamylose with 26 glucose rings (Przylas *et al*., 2000)

Amylomaltase was first found in *Escherichia coli*, but seems to be distributed in various bacterial species with different physiological functions. In a number of micro-organisms, e.g. *E. coli*, amylomaltase is expressed with glucan phosphorylase and is

proposed to be a member of the maltooligosaccharide transport and utilization system (Schwartz, 1987). In other microorganisms, e.g. *Aquifex aeolicus*, the presence of an amylomaltase-encoding gene is highly correlated with that of α-1, 4-glucan-branching enzyme and glycogen phosphorylase, which suggests a role for amylomaltase in glycogen synthesis (Kaper *et al*., 2004). A similar enzyme, termed disproportionating enzyme (D-enzyme), is present in plants. The physiological role of D-enzyme, which is located in plastids, has not been clarified, but the enzyme is assumed to be involved in starch turnover (Takaha and Smith, 1999).

The main application of amylomaltase is to utilize for the industrial production of LR-CDs which are highly soluble in water and can form inclusion complexes with several guest molecules, and it is expected that large cycloamyloses will be used in the food, pharmaceutical, and chemical industries (Terada *et al*., 1999). However, the intermolecular transglycosylation of amylomaltase has not much been studied. Other application of amylomaltase is to use for the production of a thermoreversible starch gel that can be used as a substitute for gelatin. When gelatinized potato starch was treated with the amylomaltase from *T. thermophilus*, a product free of amylose and containing amylopectin with shortened and elongated side chains was obtained. This product could be dissolved in water and formed after heating and cooling a firm gel. The gel could be dissolved again by a new heating step (Figure 1.13) (Binnema and Euverink, 2004).

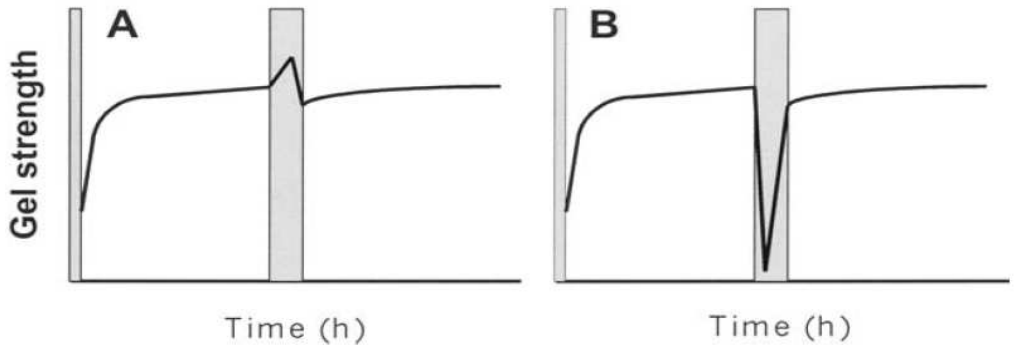


Figure1.13 Rheological behaviour of untreated potato starch (A) and amylomaltase- treated potato starch (B) illustrating the thermoreversible gelling properties of the latter (Grey bars indicate heating steps) (Kaper *et al*., 2004)

1.5 Transglycosylation activity for the synthesis of maltosylsucrose

Maltosyl fructofuranoside or called maltosylsucrose (G_nF, G=glucose, F=fructose), is found in trace amount in honeydew (produced by insect) (Gray and Fraenkel, 1953), fungi (Tsuchida *et al*., 1966), plant (Keller and Wiemken, 1982) and cyanobacteria (Fischer *et al*., 2006). It is also synthesized by the transglycosylation reaction with various glucosyl donors and sucrose acceptor using different enzymes obtained from microbes and honey. Two industrial processes for the synthesis of coupling sugar have been patented using CGTases from *Bacillus macerans* (Okada et. al, 1974) and *Thermoanaerobacter* (Pedersen, 1992) when both enzymes used starch as donor and sucrose as acceptor (Figure 1.14) (Martín *et al*., 2004).

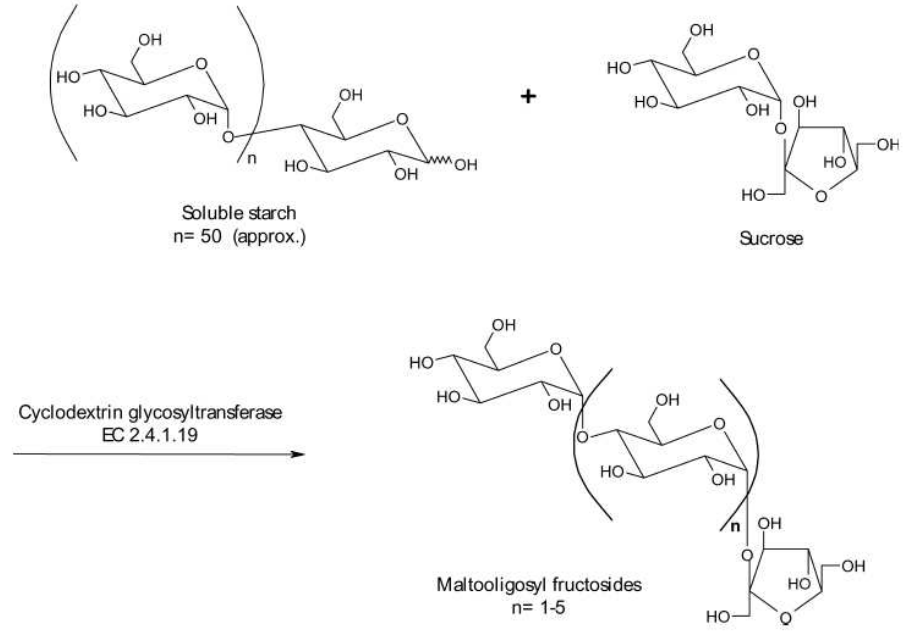


Figure 1.14 Acceptor reaction using soluble starch as donor and sucrose as acceptor catalyzed by CGTase (Martín *et al*., 2004)

A mixture of maltooligosyl sucrose with a degree of polymerization of 3–7 is currently being commercialized as coupling sugar, a non-reducing sugar with anticariogenic property (Martín *et al*., 2004). The relative sweetness of maltosylsucrose is about 50-60% of that of sucrose (Oku and Nakamura, 2002) and used as viscosity control agent, with its higher chemical and thermal stability as compared to other sweeteners containing reducing sugars being notable (Plou *et al*.,

2002). Moreover, other health benefits of maltosylsucrose have been reviewed e.g. improvement of gastrointestinal conditions, promotion of mineral absorption, stimulation of the immune system, etc. (Crittenden and Playne, 1996; Swennen *et al*., 2006).

Maltosylsucrose has been reported as a product of several enzymes acting on various glucosyl donors and sucrose acceptor via transglycosylation reaction. Examples of its synthesis are as follow.

(a) White and Maher (1953) reported the synthesis of maltosylsucrose from sucrose by the activity of honey invertase on sucrose alone. The enzyme acted on melezitose, raffinose, sucrose and maltose, but was inhibited by glucose. The structure of the product from sucrose was mainly a trisaccharide, α-D-glucopyranosyl)-(1, 4)-α-D-glucopyranosyl- (1, 2)-β-fructofuranoside.

(b) Okada *et al.* (1974) reported the synthesis of maltosylsucrose by an α-CGTase from *Bacillus macerans.* The enzyme produced the sweetener with free of reducing sugars from a mixture of dextrin and sucrose when subjected to the action of CGTase. It was proved that glucose moieties of dextrin chain are bound to glucose moiety in sucrose and the fructose moiety being terminal. The composition of the products was varied depending on the ratio of dextrin to sucrose and the transferred saccharides having D.P. of less than 9 were obtained at 75% yield.

(c) Pedersen (1992) reported that the enzymatic tranglycosylation of CGTase from *Thermoanaerobacter* (α/β CGTase) was utilized to produce maltosylsucrose. This thermostable CGTase was used both for starch liquefaction and for subsequent transglycosylation, the reaction was incubated with amylopectin and sucrose as substrate at high temperature. Glucosyl sucrose (G_2F) was the main product when incubated amylopectin and sucrose as substrate with CGTase from *Thermoanaerobacter sp.* at 90°C.

(d) Okada *et al*. (2002) reported the synthesis of maltosylsucrose using the α-glucosidase from *Bacillus* sp. SAM 1606. They carried out site-specific mutagenesis study, in which an amino acid residue Gly273 and Thr272 of α-glucosidase were replaced by other amino acid residues. The wild type and other mutated enzymes produced α-D-glucopyranosyl-(1, 6)-α-D-glucopyranosyl-(1, 2)-β-fructofuranoside (or called theanderose) as the major transfer product along with other trisaccharides (i.e. α-D-glucopyranosyl-(1, 6)-α-fructopyranoside-(2, 1)-β-glucopyranoside (isomelezitose) and α-D-glucopyranosyl-(1, 4)-α-fructopyranoside-(2, 1)-β-glucopyranoside. However, the yields of theanderose produced by these mutants were lower than that with the wild type.

(e) Lee *et al*. (2003) reported the preparation of maltosylsucrose by the activity of maltogenic amylase from *Bacillus stearothermophilus*. The transglycosylation reaction was conducted with maltotriose and sucrose as the donor and acceptor. The structure of the major transfer product were determined to be two tetrasaccharides, α-D-glucopyranosyl-(1, 4)-α-D-glucopyranosyl-(1, 6)-α-glucopyranosyl-(1, 2)-β-fructopyranoside and α-D-glucopyranosyl-(1, 4)-α-D-glucopyranosyl-(1, 6)-α-fructopyranoside-(2, 1)-β-glucopyranoside.

(f) Monthieu *et al.* (2003) reported the purification and characterization of maltosylsucrose produced by CGTase from *Thermoanaerobacter*. The capacity of CGTase for using β-CD as glucosyl donor and transferring it to sucrose molecule by coupling reaction, which the DP of different products was limited to value of 8 and this allowed to purify all of them by size exclusion chromatography. When the products were analyzed with mass spectrometry and NMR analysis, they consisted of linear oligosaccharide of various DP bound to the glucose moiety of a sucrose molecule by α (1, 4) linkage.

(g) Martín *et al.* (2004) reported the synthesis of maltosylsucrose by immobilized CGTase from *Thermoanaerobacter*. The reaction employed

soluble starch as donor and sucrose as acceptor, a series of maltosylsucrose product is subsequently formed with the maltooligosaccharide of various DP bound to glucose moiety of a sucrose molecule by α-(1, 4)-linkage in which the size of products with DP up to 3-8 were also observed and the main products were maltosyl fructose (G_2F), maltotriosyl fructose (G_3F) and maltotetraosyl fructose (G_4F). The yield of maltosylsucrose from immobilized CGTase obtained (92%) was higher than that produced by soluble CGTase (80%).

(h) Soro *et al.* (2007) reported the enzymatic synthesis by α-glucosidase from the digestive juice of a snail to produce α-(1, 4) maltosylsucrose from sucrose substrate only. The enzyme showed various substrate specificity, was active on sucrose, pNP-α-D-gluco glucopyranoside and maltose, but it was unable to hydrolyze raffinose, and polymers such as amylose, inulin and cellulose. The transfer products were observed from DP 3 to 8 and the main product was maltosyl fructose (G_2F).

From literature review, the transferred products and yields were different depending on the type of enzyme used to catalyze the transglycosylation reaction. Our research group has carried out research on 4-α-GTase comprising of CGTase and amylomaltase, and utilization of these two enzymes in the synthesis of cyclodextrin. *Paenibacillus* sp. RB01 was isolated from hot spring area at Ratchaburi province, Thailand with a high CGTase (β-CGTase) activity. The enzyme was able to produce small ring cyclodextrins (Yenpetch, 2002) and to catalyze transglycosylation reaction with glucoside acceptor (Aramsangtienchai, 2007). Furthermore, a recombinant amylomaltase from *Corynebacterium glutamicum* ATCC 13032 was expressed in *Escherichia coli* BL21 (DE3) and was fractionated with disproportionation activity. The enzyme was utilized for the synthesis of large ring cyclodextrins (Srisimarat *et al*, 2010). The focus of this research is then on the use of CGTase and recombinant amylomaltase in transglycosylation reaction with glucosyl donors and sucrose acceptor for the synthesis of maltosylsucrose. Transglycosylation ability will be compared and the ratios of products will be analyzed.

The objectives of this thesis

1. Synthesis of maltosylsucrose using various glucosyl donors and sucrose acceptor catalyzed by CGTase and recombinant amylomaltase.
2. Selection of the most suitable donor and optimization of the transglycosylation reaction.
3. Larger scale preparation, isolation and characterization of the transglycosylation products.
4. Determination of biological properties of the transglycosylation products.

CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Autoclave: Model HV-110, Hirayama Manufacturing Cooperation, Japan

Autopipette: Pipetman, USA

Balance: AB204-S, Mettler Toledo, Switzerland

Balance: PB303-S, Mettler Toledo, Switzerland

Centrifuge, refrigerated: Model Avanti^{TM} J-30I, Beckman Instrument, USA

Centrifuge, refrigerated: Model Mikro 22R, Andreas Hettich GmbH & Co. KG, Germany

Centrivap Concentrator: Model 79700-01, Labconco Corporation, USA

Electrophoresis unit: Model Mini-protein II Cell, Bio-Rad, USA

Evaporator: Rotavapor R-200, BÜCHI, Switzerland

Fraction collecter: Frac-920, Amersham Biosciences, USA

High Performance Liquid Chromatography: Model LC-3A Shimadzu, Japan

Hitrap affinity (HisTrap ^{FF}TM) column: GE Healthcare, UK

Incubator shaker: innova^{TM} 4080, New Brunswick Scientific Co., Inc., USA

Incubator: Haraeus, Germany

Laminar flow: Thermo Scientific Forma® Class II, Thermo Fisher Scientific Inc., USA

Lyophilizer: FreeZone 2.5 Liter Benchtop Freeze Dry System, Labconco Corporation, USA

Mass spectrometer: MicrOTOF, Bruker, Germany

Oven: Model 8150, Contherm, New Zealand

pH meter: Seven Easy, Mettler Toledo, Switzerland

Rezex RSO-Oligosaccharide column, Phenomenex, Inc, USA

TLC plates: Silica gel 60 F_{254}, Merck, Germany

UV-VIS Spectrophotometer: DU 650 Spectophptometer, Beckman, USA

Vortex: Model K-550-GE, Scientific Industries, USA

Water bath: Memmert, Germany

2.2 Chemicals

Acrylamide: Amersham Biosciences, USA

Agar: Scharlau, Spain

Ampicillin: Sigma, U.S.A

Bacto™ Brain Heart Infusion: BD, USA

Beef extract: Biomark Laboratories, India

Bio-Gel P-2 Gel: Fine, Bio-Rad Laboratories, USA

Bovin serum albumin (BSA): Sigma, U.S.A.

n-Butanol: Carlo Erba, Italy

Coomassie brilliant blue G-250, R-250: Sigma, USA

β-Cyclodextrin: Sigma, USA

Dialysis tubing: Sigma, USA

Ethanol: Merck, Germany

D(-)Fructose: Sigma, USA

Glycerol: Sigma, U.S.A.

Glycine: Sigma, U.S.A

D(+)-Glucose: Sigma, U.S.A.

Hydrochloric acid: J.T. Baker, USA

Imidazole: Fluka, Switzerland

Iodine: Baker chemical, U.S.A.

Isopropylthiogalactoside IPTG: United states Biological, U.S.A.

LMW-SDS Marker: GE Healthcare, USA

Maltoheptaose: Sigma, U.S.A

2-Mercaptoethanol: Bio Basic Inc, Canada

Methanol: Merck, Germany

Peptone from casein: Scharlau, Spain

Phenolpthalein: Scharlau, Spain

Di-Potassium hydrogen phosphate: Univar, Australia

Potassium dihydrogen phosphate: Scharlau, Spain

Potassium iodine: Mallinckrodt, U.S.A.

Pyridine: Carlo Erba, Italy

Sodium chloride: Carlo Erba, Italy

Sodium dodecyl sulfate: Sigma, USA

Sodium hydroxide: BDH, Canada

Soluble starch, potato: Scharlau, Spain

Standard molecular weight marker protein:

D(+)-Sucrose: Fluka, switzerland

Sulphuric acid: J.T. Baker, USA

TEMED (N, N, N', N'-tetramethylene-ethylenediamine): Fluka, Germany

Tris (hydroxylmethyl)-aminomethane: USB, USA

Yeast extract: Scharlau, Spain

Pea starch was kindly provided by Emsland-Stärke GmbH (Emlichheim,Germany)

Raw tapioca starch (Dragon Fish) and corn starch (Maizena) were locally purchased

2.3 Bacteria

Paenibacillus sp. RB01 with CGTase activity was previously isolated from hot spring area at Ratchaburi province, Thailand (Tesana, 2001) and was used for the production of CGTase.

pCGAM was obtained by inserting the amylomaltase gene from *Corynebacterium glutamicum* ATCC 13032 into *Escherichia coli* BL21 (DE3) using the expressed plasmid pET-19b (Srisimarat *et al*, 2010)

Streptococcus mutans ATCC 25175 was obtained from Oral biology Research Center, Faculty of density, Chulalongkorn University.

2.4 Media Preparation

2.4.1 Medium I

Medium I was consisted of 0.5% (w/v) beef extract, 1.0% (w/v) peptone, 0.2% (w/v) Nacl, 0.2% (w/v) yeast extract and 1.0% (w/v) soluble starch. The pH of medium was adjusted to 7.2 with 1 M NaOH. For solid medium, 1.5% (w/v) agar was added

2.4.2. Horikoshi's medium

Medium for enzyme production, slightly modified from Horikoshi (1971) by Rutchtorn (1993) contained 1.0% (w/v) soluble starch, 0.5% (w/v) peptone, 0.5% (w/v) yeast extract, 0.1% (w/v) K_2HPO_4, 0.2% (w/v) $MgSO_4.7H_2O$ and 0.75% (w/v) Na_2CO_3. The pH of the medium was 10.1-10.2.

2.4.3 LB medium

LB medium consisted of 1.0% (w/v) tryptone, 0.5% (w/v) yeast extract and 0.5% (w/v) NaCl. For solid medium, 1.5% (w/v) agar was added.

2.5 Cultivation of bacteria

2.5.1 Starter inoculums

Paenibacillus sp. RB01, steaked on solid Medium I and incubated at 37°C for

18 hours. One loop was inoculated into liquid Medium I and grown until A_{660} reached 0.3-0.5.

pCGAM, streaked on solid LB medium containing 100 µg/ml ampicillin and incubated at 37°C for 16-18 hours. One colony was inoculated into 50 ml liquid LB medium and incubated at 37°C for 14-16 hours.

2.5.2 Enzyme production

Starter inoculum of *Paenibacillus* sp. RB01 was 1.0% (v/v) transferred into 300 ml of Horikoshi's medium in 1 liter of Erlenmeyer flask and cultivated at 40°C for 72 hours. Cells were removed by centrifugation at 3,800 x g at 4°C. Culture broth with crude CGTase was collected and kept at 4°C for further purification.

Starter inoculums 1.0% (v/v) of pCGAM was transferred into 300 ml of LB medium containing ampicillin (100 µg/ml) and cultivated at 37°C and grown until A_{660} reached 0.4-0.6. IPTG was added to culture to make 0.4 mM concentration and the cells were allowed to grow for 2 hours. After that, cells were collected by centrifugation at 3,800 x g for 10 minutes and washed with 0.85% (w/v) NaCl and extraction buffer (50 mM KPB, pH 7.4 containing 0.1 mM PMSF, 0.01% (v/v) β-mercaptoethanol, 1 mM EDTA), respectively. The cells were then collected by centrifugation at 3,800 x g for 10 minutes. The collected cells were resuspended in extraction buffer (1 g of cell weight was resuspended in 2.5 ml of extraction buffer) before subjected to sonication. Unbroken cells and cell debris were removed by centrifugation at 17,000 x g for 30 minutes. The crude enzyme was collected and dialyzed against 20 mM phosphate buffer, pH 7.4 at 4°C.

2.6 Partial purification of enzyme

2.6.1 Partial purification of CGTase

CGTase was purified from the culture broth of *Paenibacillus* sp. RB01 using the starch adsorption method (Charoensakdi *et al*, 2007).

Corn starch was oven dried at 120°C for 30 minutes and cooled to room temperature. It was then gradually sprinkled into stirring crude CGTase broth to make 5% (w/v) concentration. After 3 hours of continuous stirring, the starch cake was collected by centrifugation at 3,800 x g for 30 minutes and washed twice with 10 mM Tris-HCl containing 10 mM $CaCl_2$, pH 8.5 (TB1). The adsorbed CGTase was eluted from the starch cake with TB1 buffer containing 0.2 M maltose (2x125 ml for 1 litre of starting broth), by stirring for 30 minutes. Eluted CGTase was recovered by centrifugation at 9,800 x g for 30 minutes. The enzyme solution was dialyzed against 50 mM phosphate buffer pH 6.0 containing 10 mM $CaCl_2$ at 4°C with 3 changes of buffer.

2.6.2 Partial purification of recombinant amylomaltase

Crude amylomaltase (containing His-tag fragment) was loaded onto a 1 ml Hitrap affinity (HisTrap ^{FF}TM) column which was previously equilibrated with 20 mM phosphate buffer containing 0.5 M NaCl and 20 mM imidazole, pH 7.4 for 5 column volume. Other proteins were washed by the same buffer 10-15 column volume. The purified enzyme was then eluted with 20 mM phosphate buffer containing 0.5 M NaCl and 500 mM imidazole, pH 7.4 (elution buffer). The column was washed with ultra pure water for 5-10 column volume before storage. The enzyme solution was dialyzed against 20 mM phosphate buffer, pH 7.4 at 4°C.

2.7 Polyacrylamide Gel Electrophoresis (PAGE)

SDS-PAGE was employed for analysis of the purified enzyme according to Bollag *et al*. (1996).

2.7.1 SDS-PAGE

The denaturing gel was carried out with 0.1% (w/v) SDS in 7.5% (w/v) separating and 5.0% (w/v) stacking gels and Tris-glycine buffer, pH 8.0 containing 0.1% (w/v) SDS was used as electrode buffer (see Appendix A). Samples to be analyzed were 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.

2.7.2 Detection of proteins

After electrophoresis, proteins on denaturing gels were visualized by Coomassie blue staining.

2.7.2.1 Coomassie blue staining

Gels were 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 1 hour. The slab gels were destained with a solution of 10% (v/v) methanol and 10% (v/v) acetic acid for 1-2 hours, followed by several changes of destaining solution until gel background was clear.

2.8 Enzyme assay

CGTase activity was determined by the starch degrading (dextrinizing) activity assay. For amylomaltase activity, iodine method was used to measure the disproportionation reaction.

2.8.1 Dextrinizing activity

Dextrinizing activity of CGTase was measured by the method of Fuwa (1954) with slight modification (Techaiyakul, 1991).

Sample (10-100 µl) was incubated with 0.15 ml of starch substrate (0.2% (w/v) soluble potato starch in 0.2 M phosphate buffer pH 6.0) at 40°C for 10 minutes. The reaction was stopped with 2 ml of 0.2 M HCl. Then 0.25 ml of iodine reagent (0.02% (w/v) I_2 in 0.2% (w/v) KI) was added. The mixture was adjusted to a final volume of 5 ml with distilled water and its absorbance at 600 nm was measured. For a control tube, 0.2 M HCl was added before the enzyme sample.

One unit of enzyme was defined as the amount of enzyme which produces 10% reduction in the intensity of the blue color of the starch-iodine complex per minute under described conditions.

2.8.2 Disproportionation activity

Amylomaltase activity was measured by the iodine method of Park *et al.* (2007) with slight modification (Srisimarat *et al.*, 2010).

Sample (0–100 µl) was incubated with 250 µl of 0.2% (w/v) soluble potato starch, 50 µl of 1% (w/v) maltose and 0.6 ml of 50 mM Tris-HCl pH 7.4 at 30°C for 10 minutes and the reaction was stopped by boiling for 10 minutes. Then 100 µl aliquots were withdrawn and mixed with 1 ml iodine solution (0.02% (w/v) I_2 in 0.2% (w/v) KI), and the absorbance at 600 nm was measured. For control tube, elution buffer was replaced for the enzyme.

One unit is defined as the amount of enzyme that produces a one percent reduction in the intensity of the color (absorbance) of the starch-iodine complex per minute under described conditions.

2.9 Protein determination

Protein concentration was determined by the Bradford's method (1976), using bovine serum albumin as a standard protein (see Appendix C)

One hundred microlitres of sample was mixed with 1 ml of protein reagent and left for 15 minutes before recording the absorbance at 595 nm. One litre of Coomassie blue reagent was the mixture of 100 mg Coomassie blue G-250, 50 ml of 95% (v/v) ethanol, 100 ml of 85% (v/v) H_3PO_4 and distilled water.

2.10 Synthesis of maltosylsucrose and detection of the products

2.10.1 Donor specificity

The transglycosylation reaction of CGTase consisting of 5% (w/v) of various glucosyl donors, 5% (w/v) sucrose and 200 U/ml (dextrinizing unit) of CGTase in 0.2 M phosphate buffer pH 6.0 was carried out at 40°C for 24 hours. Various glucosyl donors for CGTase reaction were potato starch (soluble), tapioca starch (soluble), maltoheptaose and β-CD**.** For amylomaltase, 2.5% (w/v) of various glucosyl donors and 2.5% (w/v) sucrose was incubated with 0.5 U/ml of amylomaltase in 50 mM phosphate buffer pH 6.0 at 30°C for 24 hours. The glucosyl donors used were raw tapioca starch, raw pea starch and raw corn starch. The transfer reaction products

were analyzed by TLC.

2.10.2 Detection of products

2.10.2.1 TLC analysis

TLC was performed on silica gel 60 F_{254} aluminium sheets using n-butanol/pyridine/water 5:4:1 (by volume) as eluent. The developed plate was dried and visualized by spraying with H_2SO_4-methanol (1:2, v/v), then heating at 110°C for 10 minutes.

2.10.2.2 HPLC analysis

The reaction mixture was centrifuged for 30 minutes at 10,000 x g and filtered by using a 0.45 µm membrane filter. Sample was then applied onto Rezex RSO-Oligosaccharide column (200 x 10.0 mm, Phenomenex, Inc., USA) connected to a refractive-index detector. The adsorbed compounds were eluted with ultra pure water at a flow rate of 0.3 ml/min. The column temperature was raised to 80°C.

2.10.3 Determination of transglycosylation efficiency

Efficiency was judged by transglycosylated product yield which was calculated from peak area of product to that of initial sucrose concentration in HPLC profile, using the equation:

$$\text{Product yield (\%)} = \frac{\text{Peak area of product}}{\text{Peak area of sucrose at } t_0} \times 100$$

2.11 Optimization of transglycosylation reaction

2.11.1 Effect of sucrose concentration

The reaction was performed by incubation of appropriate concentration of CGTase (200 U/ml) or recombinant amylomaltase (0.5 U/ml) with various sucrose concentrations (for CGTase: 0.025, 0.05, 0.10, 0.25, 0.50, 1.0, 2.5, 5.0 and 10.0%, w/v; for amylomaltase: 0.25, 0.50, 1.0, 1.5, 2.0 and 2.5%, w/v) and with 5% (w/v)

soluble tapioca starch as a glucosyl donor for CGTase and 2.5% (w/v) raw tapioca starch for amylomaltase, at 40°C for 24 hours for CGTase reaction and at 30°C for 24 hours for amylomaltase reaction. The reaction was stopped by boiling for 10 minutes, and then analyzed by HPLC.

2.11.2 Effect of donor concentration

The reaction was performed by incubation of appropriate concentration of CGTase (200 U/ml) or recombinant amylomaltase (0.5 U/ml) with various donor concentrations (for CGTase: soluble tapioca starch were 0.25, 0.50, 1.0, 2.5, 5.0, 10.0, 15.0, 20.0, 25.0 and 30.0%, w/v; for amylomaltase: 0.25, 0.50, 1.0, 1.5, 2.0, 2.5 and 3.0%, w/v) with optimum sucrose concentration, at 40°C for 24 hours for CGTase reaction and at 30°C for 24 hours for amylomaltase reaction. The reaction was stopped by boiling for 10 minutes, and then analyzed by HPLC.

2.11.3 Effect of enzyme concentration

The reaction was performed by incubation of various amount of CGTase (6.25, 12.5, 25.0, 50.0, 100, 200, 300, 400 and 500 U/ml dextrinizing unit) and amylomaltase (0.25, 0.5, 0.75, 1.0, 3.0, 5.0, 7.0, 9.0 and 12.0 U/ml disproportination unit) with optimum sucrose and donor concentrations in 0.2 M phosphate buffer, pH 6.0 at 40°C for 24 hours for CGTase and in 50mM phosphate buffer, pH 6.0 at 30°C for 24 hours for amylomaltase. The reaction was stopped by boiling for 10 minutes, and then analyzed by HPLC.

2.11.4 Effect of incubation time

The reaction was performed by incubation of appropriate concentrations of CGTase and amylomaltase with optimum sucrose and donor concentrations for various times (for CGTase: 6, 12, 24, 36 and 48 hours; for amylomaltase: 6, 12, 24, 36, 48 and 72 hours). The reaction was stopped by boiling for 10 minutes, and then analyzed by HPLC.

2.12 Large scale preparation and isolation of glucosylated products

In the initial experiments, to prepare maltosylsucrose and to determine transglycosylation efficiency (section 2.10) and to optimize the transglycosylation reaction (section 2.11), small scale reaction mixture of 0.5 ml was used. To prepare higher amount of products for characterization, larger scale preparation (25 ml) of reaction mixture using optimum condition for transglycosylation as obtained from section 2.11 was performed. The reaction mixture was applied onto Biogel-P2 column (1.80x80 cm) equilibrated with distilled water and the transfer products were eluted by distilled water with the flowrate of 15 ml/h (fraction size 1 ml). The fractions were detected by phenol-sulfuric method. The fractions containing sugar were concentrated with a Centrivap Concentrator at 45°C. The concentrated fractions were determined by HPLC.

2.12.1 Sugar determination

Carbohydrate content was determined by the phenol-sulfuric acid method of Dubois *et al*. (1956) with slight modification.

One hundred microlitres of sample solution was mixed with 2.0 ml of conc. H_2SO_4 and 0.5 ml of 5% (w/v) phenol, kept for 10 minutes, cooled, measured at 490 nm.

2.13 Characterization of maltosylsucrose

2.13.1 Mass Spectrometry

Mass analysis of major transfer products was performed by mass spectrometry. Electrospray Ionization-Time of Fight 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: water (1:1, v/v) was used as solvent.

2.14 Determination of properties of maltosylsucrose

Monitoring biological properties of maltosylsucrose products in inhibition of the activity of glucosyltransferase (GTase) from *Streptococcus mutans* was performed.

2.14.1 Starter inoculums

S. mutans was streaked on a solid brain heart infusion, BHI (3.7% (w/v) consisted of 0.77% (w/v) calf brains infusion, 0.98% (w/v) beef heart infusion, 0.1% (w/v) proteose peptone, 0.2% (w/v) dextrose, 0.5% (w/v) sodium chloride and 0.25% (w/v) disodium phosphate with 1.5% (w/v) agar) and incubated at 37°C for 3 days. One colony was inoculated into liquid BHI and grown for 20 hours at 37°C.

2.14.2 GTase production

GTase preparation was partially purified by the method of Shimamura *et al.* (1982) with slight modification.

Starter inoculum 1.0% (v/v) of *S. mutans* was transferred into 300 ml of BHI broth and cultivated at 37°C for 18 hours. The supernatant was obtained by centrifugation at 3,800 x g at 4°C for 10 minutes. Cold absolute ethanol was added to a final concentration of 40% (v/v). After standing at 4°C overnight, the precipitate formed was collected by centrifugation at 3,800 x g at 4°C for 15 minutes and suspended in distilled water (20 ml for 1.8 litre of starting broth) and the suspension was adjusted to pH 6.8 with diluted ammonium solution and then dissolved by stirring for 1 hour. Insoluble material was removed by centrifugation at 15,000 x g for 30 minutes and the supernatant was dialyzed against 50 mM potassium phosphate buffer (pH 6.8).

2.14.3 The inhibitory effects of maltosylsucrose on cariogenicity

The synthesis of water-insoluble glucan and acid fermentation were measured by the method of Lee *et al.* (2003), plaque assay was measured by the method of Hirasawa *et al*. (1980) and cell aggregation was measured by the method of Gibbons and Fitzgerald (1969) with slight modification.

2.14.3.1 The inhibitory effects of maltosylsucrose on the synthesis of water insoluble glucan

Twenty five microlitres of pre-cultured *S. mutans* was inoculated into 5 ml of a BHI broth containing 40 mg of sucrose or the mixture of maltosylsucrose or commercial coupling sugar (sugar concentrations were compared with standard sucrose by phenol-sulfuric method; see Appendix D). The bacterium was cultured at 37°C for 24 hours in a test tube. The supernatant of reaction mixtures was discarded and the synthesized glucan was washed with distilled water and dissolved in 0.5M NaOH. Water-insoluble glucan was assayed by monitoring the optical density at 550 nm.

2.14.3.2 The inhibitory effects of maltosylsucrose on the acid fermentation

The formation of acid by *S.mutans* was assayed by measuring the pH of the culture broth. Twenty five microlitres of pre-cultured *S. mutans* was added to new BHI broth containing 17 mg of sucrose or the mixture of maltosylsucrose or commercial coupling sugar. The pH of the aliquots was measured using a digital pH meter after 60 hours incubation at 37°C.

2.14.3.3 The inhibitory effects of maltosylsucrose on the plaque formation

The ability of *S. mutans* strain to form plaque was investigated by determining the amount of adherent material on glass surfaces. Twenty five microlitres of pre-cultured *S. mutans* was added to a preweighed culture tube with

new BHI broth containing 50 mg of sucrose or the mixture of maltosylsucrose or commercial coupling sugar and incubated for 24 hours. The preweighed culture tubes were washed two times with distilled water and once with ethanol (100%) to remove nonadherent material and dried, and the dry weight of the adherent material was determined.

2.14.3.4 The inhibitory effects of maltosylsucrose on cell aggregation

Cell suspension of *S. mutans* was prepared from 18 hours culture grown in BHI broth. The cells was harvested by centrifugation, washed three times in saline and suspended to an optical density of 1.0 (540 nm) in 0.2 M glycine-NaOH buffer, pH 8.5. To 0.4 ml of diluted cells, 0.2 ml of sucrose or the maltosylsucrose mixture or commercial coupling sugar (all at 1 mg/ml) was added and the reaction mixture was incubated for 18 hours at 37°C. The suspensions were scored as 0 (no agglutination) to 4+ (marked agglutination). Control cell suspensions incubated without additives were always included.

2.14.4 The inhibitory effects of maltosylsucrose on GTase activity

The formation of water-insoluble glucan was measured by the method of Shouji *et al* (2000).

In the assay of GTase activity, in a total volume of 1.0 ml consisted of 0.1 M acetate buffer (pH 5.5), 15.4 mg sucrose and/or 0-61.6 mg of the maltosylsucrose mixture or commercial coupling sugar and 100 µl of partial purified GTase. The mixture was incubated at 37°C for 20 minutes and then heat-inactivated. The turbid materials were precipitated by centrifugation (15,000 x g for 5 minutes) and washed twice with distilled water. The total amount of water-insoluble glucan was measured by the phenol sulfuric acid method and expressed as glucose equivalent (µmol glucose/min) (see Appendix E).

One unit of enzyme is defined as the amount of enzyme catalyzing the incorporation of 1 µmol of glucose from sucrose into glucan per minute.

CHAPTER III

RESULTS

3.1 Partial purification of enzymes

3.1.1 Partial purification of CGTase

The bacteria *Paenibacillus* sp. RB01 was cultivated in Horikoshi's medium containing 1.0% (w/v) soluble starch incubated at 40°C for 72 hours. The cells were separated by centrifugation and supernatant which contained crude CGTase was collected. The crude CGTase was partially purified by starch adsorption method. The enzyme activity was determined by dextrinizing assay and protein concentration was determined by Bradford's method. The purification fold and recovery of CGTase of 22.9 fold and 56.1 percent yield were obtained as shown in Table 3.1.

3.1.2 Partial purification of recombinant amylomaltase

The recombinant *E.coli* cells, pCGAM, was cultivated in LB medium containing 100 μg/ml ampicillin at 37°C and the expression of recombinant amylomaltase was induced by the addition of 0.4 mM IPTG. After induction, cells were collected by centrifugation and cells were lyzed by sonication before subjected to centrifugation in which crude amylomaltase was collected in supernatant. The crude amylomaltase was partially purified by HisTrap affinity column. The enzyme activity was determined by disproportionation activity. The purification fold and recovery of amylomaltase of 15.4 fold and 47.3 percent yield were obtained, as shown in Table 3.2.

The purity of partial purified CGTase and recombinant amylomaltase was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). For CGTase, the molecular weight was estimated to be ≈ 66 kDa which corresponded to previous report (Yenpetch, 2002) (Figure 3.1). In addition, the molecular weight of recombinant amylomaltase was approximately 84 kDa and was also in good agreement with that reported by Srisimarat *et al*. (2010) (Figure 3.2).

Table 3.1 Partial purification of CGTase from *Paenibacillus* sp. RB01

Step	Volume (ml)	Activity* (U/ml)	Protein (mg/ml)	Specific activity (U/mg protein)	Purification (fold)	Yield (%)
Crude CGTase	1600	52.4	0.609	86.0	1	100
Starch adsorbed	94	500.2	0.254	1969	22.9	56.1

* Dextrinizing activity

Table 3.2 Partial purification of recombinant amylomaltase

Step	**Volume (ml)**	**Activity* (U/ml)**	**Protein (mg/ml)**	**Specific activity (U/mg protein)**	**Purification (fold)**	**Yield (%)**
Crude amylomaltase	14	47.6	24.8	1.92	1	100
Histrap FF column	6	52.5	1.77	29.6	15.4	47.3

* Disproportionation activity

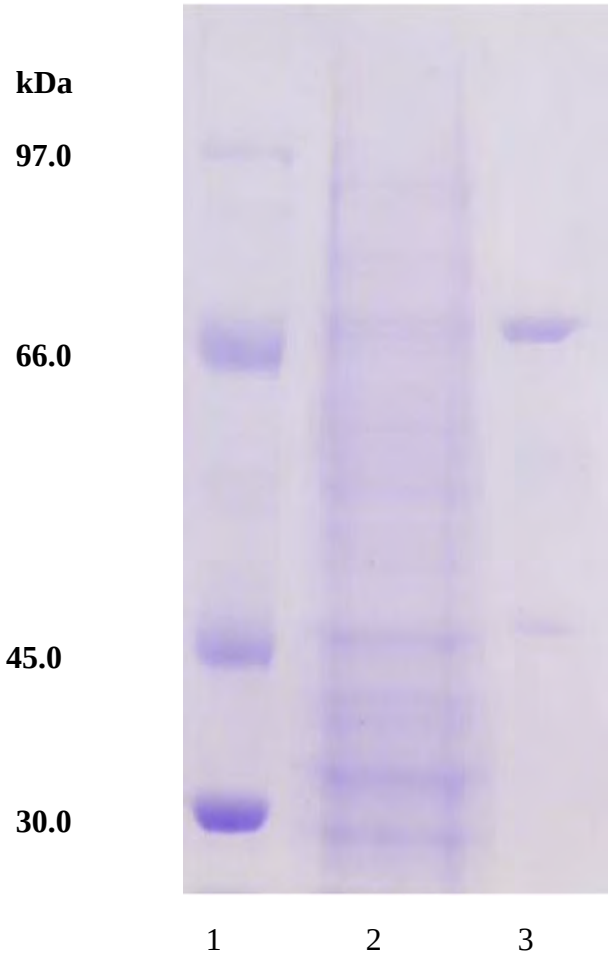


Figure 3.1 **SDS-PAGE of crude and partial purified CGTase**

Lane 1: Protein molecular weight markers
Phosphorylase b (97 kDa), Albumin (66 kDa), Ovalbumin (45 kDa) and Carbonic anhydrase (30 kDa)

Lane 2: Crude enzyme (15 µg)

Lane 3: Partial purified CCTase (3 µg)

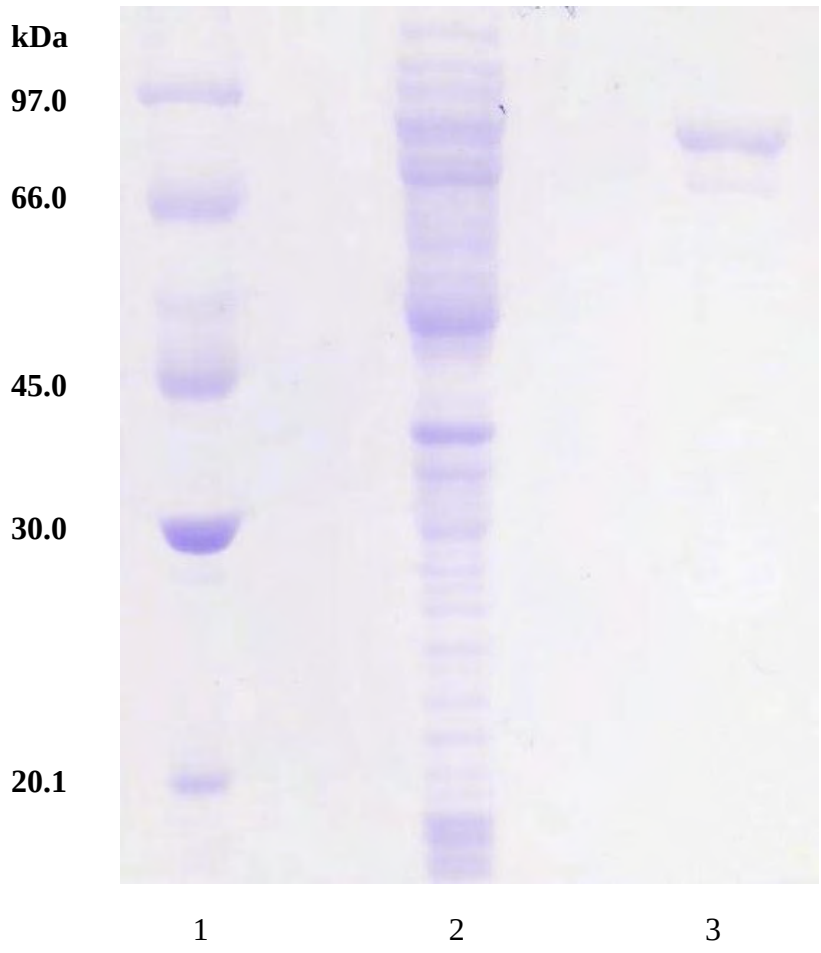


Figure 3.2 **SDS-PAGE of crude and partial purified amylomaltase**

Lane 1: Protein molecular weight markers
Phosphorylase b (97 kDa), Albumin (66 kDa), Ovalbumin (45 kDa), Carbonic anhydrase (30 kDa) and Trypsin inhibitor (20.1 kDa)

Lane 2: Crude enzyme (15 µg)

Lane 3: Partial purified amylomaltase (3 µg)

3.2 Synthesis of maltosylsucrose by CGTase and detection of the products

3.2.1 Donor specificity

Donor specificity for CGTase was determined by varying the types of donor. After incubation of CGTase (200 U/ml) with 5% (w/v) sucrose and 5% (w/v) glucosyl donor (potato starch (soluble), tapioca starch (soluble), beta-CD or maltoheptaose) at 40°C for 24 hours, reaction products were detected by TLC (Figure 3.3). From TLC chromatogram, the migration of standard sugars in this TLC system was in the order of G1 to G7 (lane a) which G1 showed the highest Rf value and sucrose was observed at a slightly lower position than G1 (lane b). The control reaction without sucrose confirmed the transfer product observed as spots on TLC (lane c, f, i and l). At initial time of incubation, only spots of sucrose and glucosyl donor used as substrate were found (lane d, g, j and m). After 24 hours of incubation, the spots expected to be maltosylsucrose were observed in the black box (lane e, h, k and n). The Rf values of all standards and products are presented in Table 3.3. All of the reaction mixture from different glucosyl donors gave four main products with similar amounts when the intensity of product spots were compared. However, soluble tapioca starch was chosen as suitable glucosyl donor because it is a cheap and abundant agro product.

3.2.2 Optimization of transglycosylation reaction

The suitable conditions for the production of maltosylsucrose were determined using soluble tapioca starch as a glucosyl donor. The reaction was performed as described in section 2.11 and the transfer products were determined by HPLC. All optimum conditions were considered from total percent yield of the transfer products (calculated from peak area of products to initial amount of sucrose (see section 2.10)). However, the optimum concentration of acceptor was considered from two profiles, % product yield as mentioned above and the amount of product which was determined from peak area.

Figure 3.3 TLC chromatogram of reaction products of CGTase incubated with sucrose and glucosyl donor (Reaction mixtures were: 5% sucrose, 5% glucosyl donor, 200 U/ml CGTase, pH 6.0, 40°C)

(a) Standard G1-G7 (10µg)

(b) Standard sucrose (10 µg)

(c) Control reaction (soluble potato starch + CGTase, no acceptor, 24 h)

(d)-(e) reaction mixture, soluble potato starch as a donor, 0 and 24 h, respectively

(f) Control reaction (soluble tapioca starch + CGTase, no acceptor, 24 h)

(g)-(h) reaction mixture, soluble tapioca starch as a donor, 0 and 24 h, respectively

(i) Control reaction (beta CD + CGTase, no acceptor, 24 h)

(j)-(k) reaction mixture, beta CD as a donor, 0 and 24 h, respectively

(l) Control reaction (maltoheptaose + CGTase, no acceptor) 24 h

(m)-(n) reaction mixture, maltoheptaose as a donor, 0 and 24 h, respectively

(o) Commercial coupling sugar

Table 3.3 Rf values from TLC analysis of standard saccharides, various donors and the reaction products of CGTase

Standard/Products			Rf
Standard	Saccharide	G1	0.76
		G2	0.68
		G3	0.60
		G4	0.52
		G5	0.45
		G6	0.40
		G7	0.35
		sucrose	0.72
	Donor	Soluble potato starch	Origin
		Soluble tapioca starch	Origin
		Beta-CD	0.61
		G7	0.35
	Commercial coupling sugar		0.76, 0.68, 0.63 0.55, 0.48, 0.42 0.37, 0.30, 0.24
Reaction products	From soluble potato starch donor	I	0.63
		II	0.55
		III	0.48
		IV	0.42
	From soluble tapioca starch	I	0.63
		II	0.55
		III	0.48
		IV	0.42
	From beta-CD donor	I	0.63
		II	0.56
		III	0.49
		IV	0.42
	From G7 donor	I	0.63
		II	0.56
		III	0.49
		IV	0.42

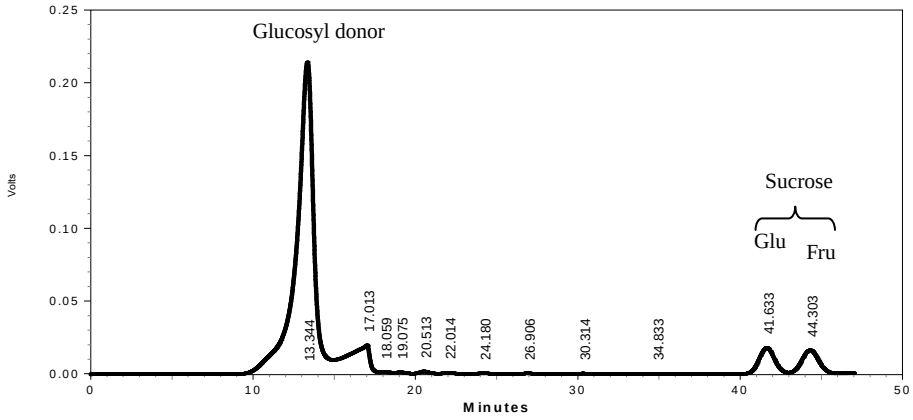
3.2.2.1 Effect of sucrose concentration

When incubating CGTase (200 U/ml) with 5% (w/v) soluble tapioca starch with various sucrose concentrations at 40°C for 24 hours, it was found that product yields varied with sucrose concentration. HPLC chromatogram of reaction products of CGTase are shown in Figure 3.4 and the transfer products were observed at Rt 19, 20, 22, 24, 26, 30, 34 min, however, the product at 22 min was also appeared in the control reaction.The percent yield of each transglycosylated product and total product yields are shown in Table 3.4 and Figure 3.5. The total product yields were significantly decreased when sucrose concentration increased to 0.05-10%. The total transglycosylation yield was found to be maximum (99.4%) when the reaction mixture was performed with 0.05% sucrose. In contrast, the amount of product (determined from peak area) increased when sucrose concentration increased to 0.05-10%. Accordingly, the optimum concentration of acceptor is at the point where the two profiles, % product yield and the amount of product are crossed over. Therefore, the optimum concentration of sucrose acceptor was 2.5% (w/v) at which the product yield obtained was 49.2%. Thus, it was chosen to be the optimum concentration.

3.2.2.2 Effect of glucosyl donor concentration

Using the conditions as mentioned in 3.2.2.1 with various soluble tapioca starch concentration, it was found that product yields varied with soluble tapioca starch concentration. The percent yield of each transfer product and total product yields are shown in Table 3.5 and Figure 3.6. The total product yield increased when glucosyl donor concentration increased from 0.25 to 20.0% and gave major products at Rt 24 and 26 min which were different from the result obtained in 3.2.2.1. A new product was observed at Rt 18 min when soluble tapioca concentration was 10-25%. The maximum product yield was 97.2% when the reaction mixture was incubated with 20% soluble tapioca starch.

A)



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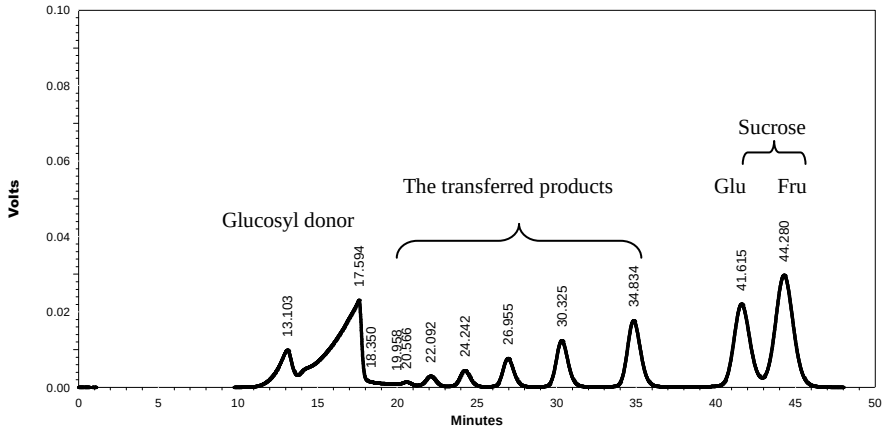


Figure 3.4 HPLC chromatogram of reaction products of CGTase

A) At 0 hour of incubation

B) At 24 hours of incubation

Table 3.4 Percent yields of transglycosylated products at different sucrose concentrations with CGTase

Sucrose concentation (%, w/v)	Product yield (%)						Total yield (%, w/v)	Peak area (x 10^6)
	Rt 19	Rt 20	Rt 24	Rt 26	Rt 30	Rt 34		
0.025	16.9	22.2	0.31	0	11.5	6.22	57.1	0.04
0.05	30.7	31.6	4.17	22.1	1.30	9.59	99.4	0.10
0.1	26.1	26.5	8.45	6.25	15.0	11.1	93.3	0.14
0.25	16.8	17.8	11.2	18.1	14.7	15.0	93.5	0.27
0.50	13.3	13.4	9.58	13.7	16.6	16.6	83.2	0.49
1.0	0	4.13	8.42	12.4	16.9	20.2	62.0	0.76
2.5	0.04	0.61	4.80	8.63	14.7	22.5	49.2	1.36
5.0	0.05	0.46	3.49	6.25	10.9	17.3	37.6	2.20
10.0	0.26	-	0.90	2.41	5.74	13.6	22.9	2.51

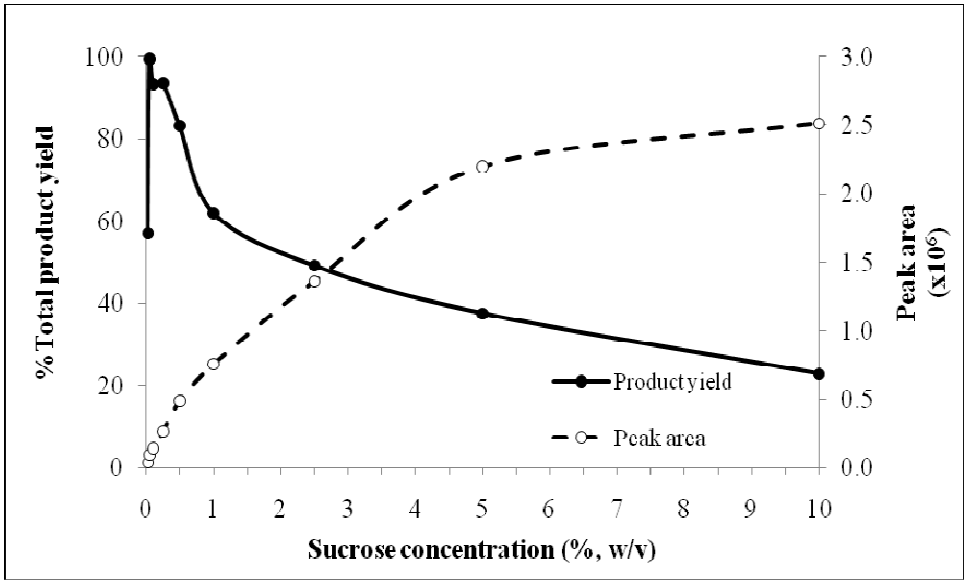


Figure 3.5 Effect of sucrose concentration on CGTase transglycosylation yield

Table 3.5 Percent yields of transglycosylated products at different glucosyl donor concentrations with CGTase

Soluble starch concentration (%, w/v)	Product yield (%)							Total yield (%, w/v)
	Rt 18	Rt 19	Rt 20	Rt 24	Rt 26	Rt 30	Rt 34	
0.25	-	-	-	0.27	0.54	1.87	10.1	10.1
0.5	-	-	-	0.56	-	3.16	10.6	14.3
1	-	-	-	0.99	2.04	5.42	14.5	22.9
2.5	0.47	1.19	-	3.40	6.09	11.6	19.3	42.0
5	-	0.35	6.02	10.2	15.3	20.0	20.5	52.0
10	4.15	6.38	7.91	12.5	14.5	16.1	16.5	78.1
15	8.24	9.86	11.3	14.8	16.0	17.0	16.7	93.9
20	9.20	12.0	13.9	15.4	17.2	16.0	13.6	97.2
25	14.7	11.0	13.8	14.7	15.9	13.0	12.4	95.4
30	-	2.77	6.13	13.0	14.7	13.9	11.6	62.1

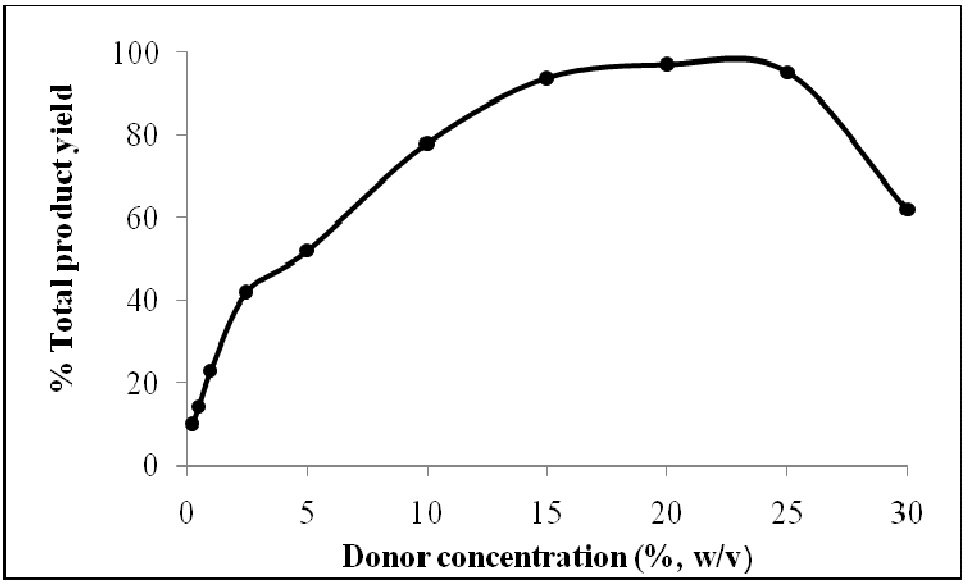


Figure 3.6 Effect of glucosyl concentration on CGTase transglycosylation yield

3.2.2.3 Effect of enzyme concentration

Using the conditions as mentioned in 3.2.2.2 with variable concentrations of CGTase, it was found that product yields varied with soluble tapioca starch concentration. The percent yield of each transfer product and total product yields are shown in Table 3.6 and Figure 3.7. The total product yield increased when CGTase concentration increased from 6.25 to 400 U/ml and gave major products at Rt 24 and 26 min. The production yield was higher than the results in 3.2.2.2 the maximum product yield obtained was 98.7% when the reaction mixture was incubated with 400 U/ml of CGTase.

3.2.2.4 Effect of incubation time

Using the conditions as mentioned in 3.3.2.3 with variable incubation time. For CGTase, it was found that product yields were clearly affected. The percent yield of each transfer product and total product yields are shown in Table 3.7 and Figure 3.8.The total product yield at 6 hours incubation was slightly low when compared with other incubation times. The maximum product yield was obtained around 98.6-98.7% when incubation time was 18-36 hours. To save on time, the 18 hours was chosen for the optimum incubation time.

Table 3.6 Percent yields of transglycosylated products at different CGTase concentrations

CGTase Concentration (U/ml)	Product yield (%)							Total yield (%)
	Rt 17	Rt 19	Rt20	Rt 24	Rt 26	Rt 30	Rt 34	
6.25	3.24	5.82	7.96	8.11	8.05	8.33	7.35	48.9
12.5	4.42	7.65	9.07	9.96	9.94	9.81	8.39	59.3
25	7.01	8.70	10.6	12.8	13.3	13.0	11.2	76.6
50	7.90	9.61	11.5	14.8	15.2	14.0	12.1	85.0
100	8.71	4.05	8.30	15.7	17.6	16.9	14.7	85.8
200	9.33	10.6	12.5	15.9	16.2	14.6	12.7	91.8
300	9.76	11.4	13.6	16.5	16.9	13.9	13.8	95.9
400	10.0	11.3	13.0	16.2	17.6	15.9	14.6	98.7
500	9.59	11.1	13.9	16.3	17.7	15.8	14.3	98.6

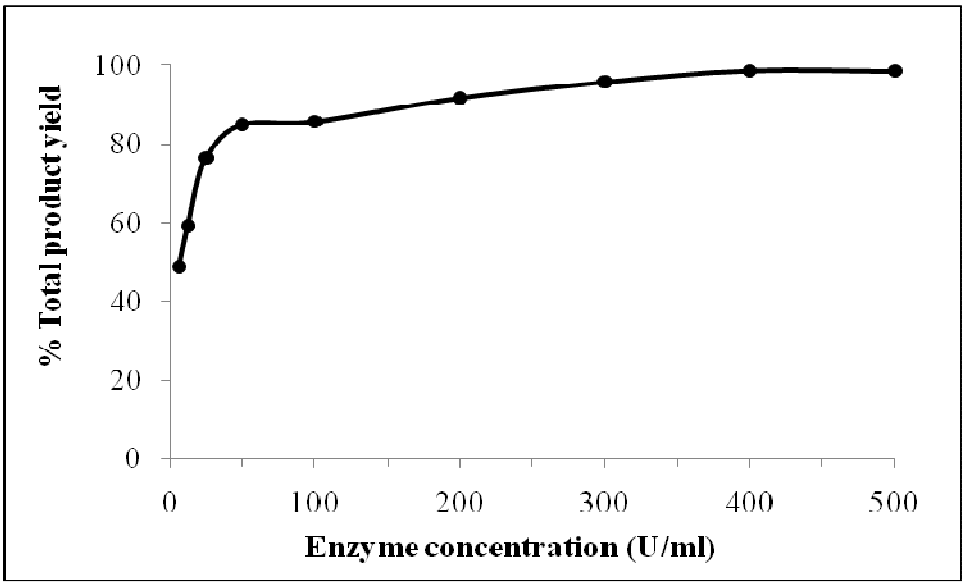


Figure 3.7 Effect of enzyme concentration on CGTase transglycosylation yield

Table 3.7 Percent yields of transglycosylated products at different incubation time with CGTase

Incubation time (hour)	Product yield (%)							Total yield (%)
	Rt 18	Rt 19	Rt 20	Rt24	Rt 26	Rt 30	Rt34	
6	7.55	10.3	10.3	15.3	16.0	15.2	13.0	87.6
12	8.39	10.9	11.2	16.9	17.3	15.5	13.3	93.4
18	9.11	11.7	11.9	17.3	18.2	16.3	14.1	98.6
24	10.1	4.64	9.03	16.7	18.8	18.3	15.6	93.2
36	10.9	6.66	8.10	17.8	20.6	18.5	16.2	98.7
48	10.9	3.19	7.53	17.5	20.6	20.2	17.8	97.7

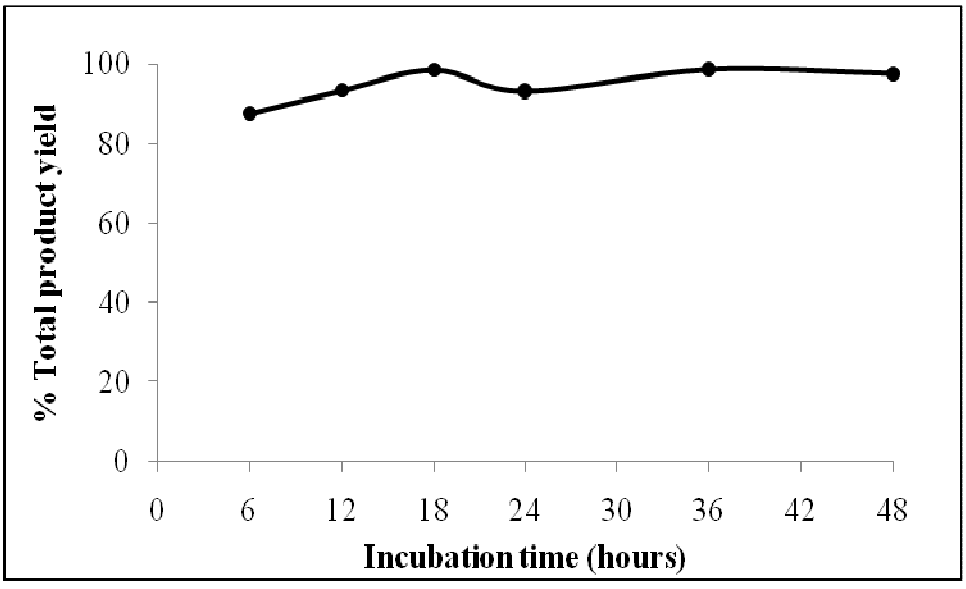


Figure 3.8 Effect of incubation time on CGTase transglycosylation yield

3.3 Synthesis of maltosylsucrose by amylomaltase and detection of the products

3.3.1 Donor specificity

Donor specificity for amylomaltase was determined by varying the types of donor. Three types of donors (raw tapioca starch, raw pea starch and raw corn starch) at 2.5% (w/v) were incubated with 0.5 U/ml of amylomaltase at 30°C for 24 hours. After incubation, the reaction products were analyzed by TLC (Figure 3.9). The control reaction without sucrose was shown on TLC that the product spots disappear except glucosyl donor (lane c, f and i). At initial incubation time, only spots of sucrose and glucosyl used as substrate were found (lane d, g and j). After 24 hours of incubation, several spots expected to be maltosylsucrose were observed in the black box (lane e, h and k). The amount of products was less than the products from CGTase. The Rf values of all standards and products are presented in Table 3.8. The intensity of the transfer products were similar and thus, were difficult to judge for the suitable glucosyl donor for amylomaltase. Transglycosylation efficiency of different donors was then investigated by HPLC using the condition as described in section 2.10. The transglycosylation product yield was determined from the ratio of peak area of product to peak area of sucrose acceptor at initial time. When raw tapioca starch, raw pea starch and raw corn starch were used as donors, the detected transglycosylation yields were 24.8, 17.8 and 15.6%, respectively. In addition, small amounts of products were found when soluble tapioca starch was used as glucosyl donor. The transglycosylation yield was only 10.6%. From HPLC results, raw tapioca starch was then chosen as a glucosyl donor for amylomaltase.

3.3.2 Optimization of transglycosylation reaction

The suitable conditions for the production of maltosylsucrose were determined using raw tapioca starch as a glucosyl donor. The reaction was performed as described in section 2.11 and the transfer products were determined by HPLC. All optimum conditions were considered from total percent yield of the transfer products (calculated from peak area of products to initial amount of sucrose (see section 2.10)). As in CGTase reaction, the optimum concentration of acceptor was considered from

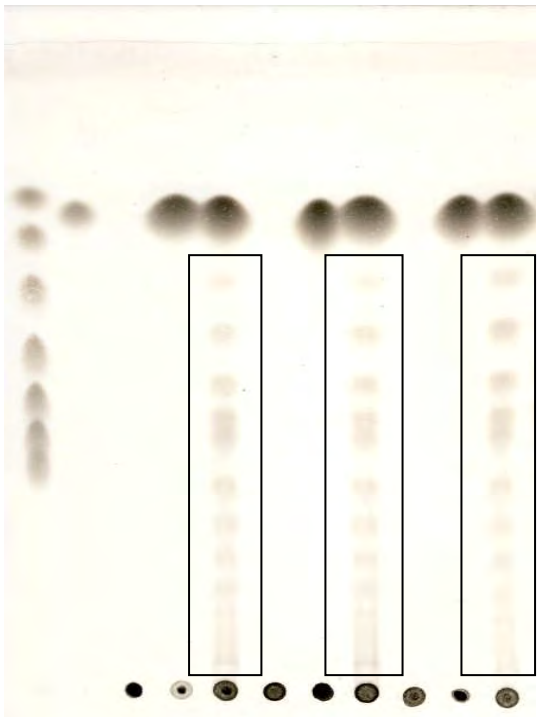


Figure 3.9 TLC chromatogram of reaction products of amylomaltase incubated with sucrose and glucosyl donors (Reaction mixtures were: 2.5% sucrose, 2.5% glucosyl donor, 0.5 U/ml amylomaltase, pH 6.0, 30°C)

(a) Standard G1-G7 (10 µg each)

(b) Standard sucrose (10 µg)

(c) Control reaction (raw pea starch + amylomaltase, no acceptor, 24 h)

(d)-(e) reaction mixture, raw pea starch as a donor, 0 and 24 h, respectively

(f) Control reaction (raw tapioca starch + amylomaltase, no acceptor, 24 h)

(g)-(h) reaction mixture, raw tapioca starch as a donor, 0 and 24 h, respectively

(i) Control reaction (raw corn starch + amylomaltase, no acceptor, 24 h)

(j)-(k) reaction mixture, raw corn starch as a donor, 0 and 24 h, respectively

Table 3.8 Rf values from TLC analysis of standard saccharides, various donors and the reaction products of amylomaltase

Standard/Products			**Rf**
Standard	Saccharide	G1	0.77
		G2	0.71
		G3	0.62
		G4	0.53
		G5	0.45
		G6	0.40
		G7	0.35
		sucrose	0.72
	Donor	Raw pea starch	Origin
		Raw tapioca starch	Origin
		Raw corn starch	Origin
Reaction products	From raw pea starch		0.64, 0.56, 0.47,
			0.42, 0.36, 0.32,
			0.25, 0.20, 0.16,
			0.12, 0.09, 0.06
	From raw tapioca starch		0.64, 0.56, 0.47,
			0.42, 0.36, 0.32,
			0.25, 0.20, 0.16,
			0.12, 0.09, 0.06
	From raw corn starch		0.64, 0.56, 0.48,
			0.42, 0.36, 0.32,
			0.25, 0.20, 0.16,
			0.12, 0.09, 0.06

two profiles, % product yield as mentioned above and the amount of product which was determined from peak area.

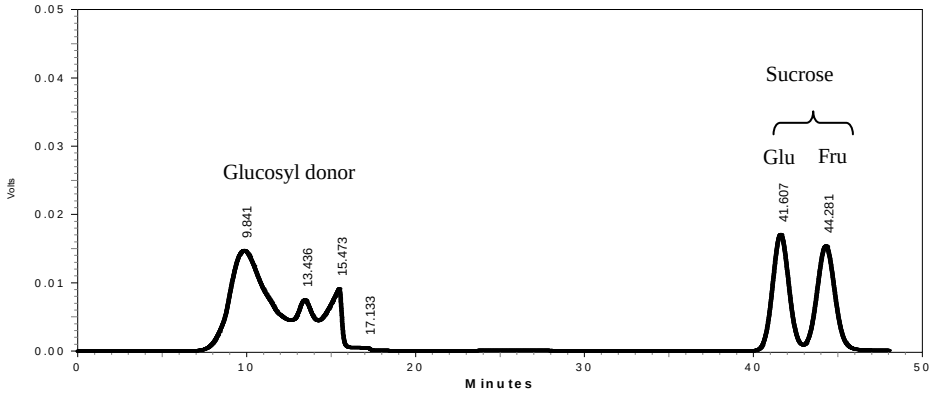
3.3.2.1 Effect of sucrose concentration

When incubating amylomaltase (0.5 U/ml) with 2.5% (w/v) raw tapioca starch and various sucrose concentrations at 30°C for 24 hours, it was found that product yields varied with sucrose concentration. HPLC chromatogram of reaction products of amylomaltase are shown in Figure 3.10 and the transfer products were observed at Rt 17, 18, 19, 20, 22, 24, 26, 30 and 34 min when the reaction mixture was incubated with 2.0 and 2.5% sucrose. The percent yield of each transfer product and total product yields are shown in Table 3.9 and Figure 3.11. The total product yield increased when sucrose concentration was increased from 0.25 to 2.0%. Also, the maximum of total product yield obtained was 11.7% when the reaction mixture was carried out with 2.0% sucrose. Hence, sucrose at 2.0% was chosen to be the optimum acceptor concentration.

3.3.2.2 Effect of glucosyl donor concentration

Using the condition as mentioned in section 3.3.2.1 with various concentration of raw tapioca starch, it was found that product yields varied with raw tapioca starch concentration. The percent yield of each transfer product and total product yields are shown in Table 3.10 and Figure 3.12. The total product yield increased when glucosyl donor concentration was increased to 0.25-2.5% and decreased when glucosyl donor concentration was more than 2.5%. Also, the maximum production yield was 18.2% when the reaction mixture was incubated with 2.5% raw tapioca starch and the major product at Rt 26 was observed.

A)



B)

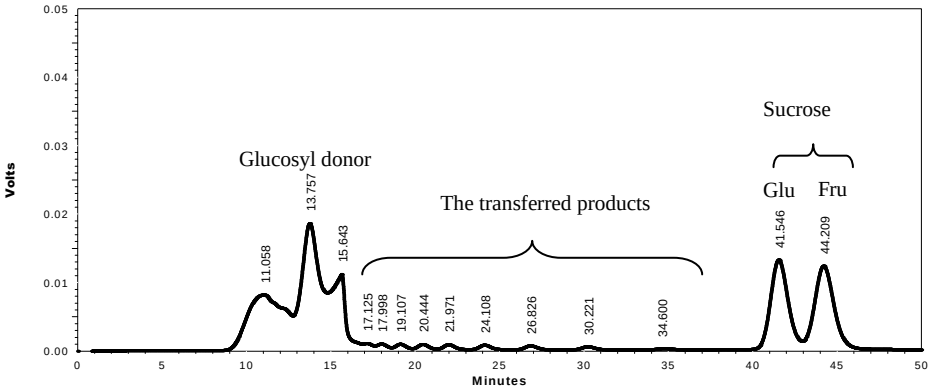


Figure 3.10 HPLC chromatogram of reaction products of amylomaltase

A) At 0 hour incubation time

B) At 24 hours incubation time

Table 3.9 Percent yields of transglycosylated products at different sucrose concentrations with amylomaltase

Sucrose concentration (%, w/v)	Product yield (%)									Total yield (%)	Peak area (x 10^5)
	Rt 17	Rt 18	Rt 19	Rt 20	Rt 22	Rt 24	Rt 26	Rt 30	Rt 34		
0.25	-	-	1.02	1.17	-	-	-	-	-	2.19	0.06
0.5	0.64	-	1.05	1.26	1.26	1.09	-	-	-	5.30	0.26
1	0.26	0.69	1.16	1.39	1.47	1.42	1.26	0.93	-	8.58	0.79
1.5	0.27	0.70	1.15	1.46	1.61	1.62	1.46	1.20	-	9.47	1.27
2	0.29	0.75	1.28	1.62	1.86	1.96	1.81	1.54	0.54	11.7	2.11
2.5	0.21	0.54	0.91	1.14	1.3	1.36	1.26	1.02	0.01	7.75	1.72

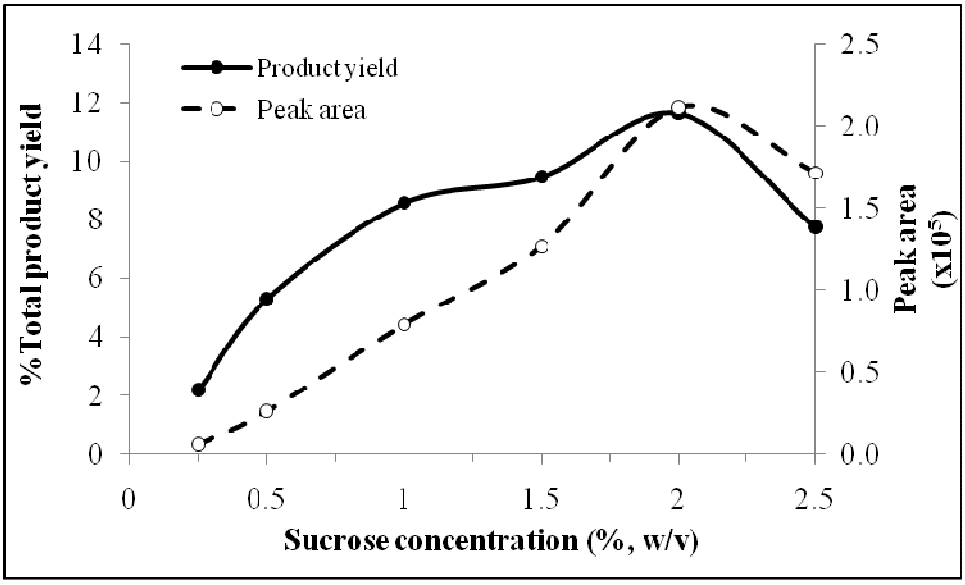


Figure 3.11 Effect of sucrose concentration on amylomaltase transglycosylation yield

Table 3.10 Percent yields of transglycosylated products at different glucosyl donor concentrations with amylomaltase

Donor concentration (%, w/v)	Product yield (%)									Total yield (%)
	Rt 17	Rt 18	Rt 19	Rt 20	Rt 22	Rt 24	Rt 26	Rt 30	Rt 34	
0.25	-	-	-	0.20	0.45	0.87	1.48	2.42	1.20	6.62
0.5	0.07	0.23	0.51	0.82	1.26	1.76	2.22	2.63	1.24	10.8
1.0	0.23	0.57	1.03	1.40	1.76	2.04	2.07	2.03	0.87	12.1
1.5	0.25	0.61	1.06	1.39	1.68	1.86	1.86	1.72	0.74	11.2
2	0.30	0.74	1.27	1.64	1.93	2.08	2.05	1.85	0.85	12.7
2.5	2.10	2.23	2.28	2.33	2.28	2.16	2.10	1.89	0.82	18.2
3.0	0.51	1.05	1.59	1.92	2.42	2.31	2.20	1.93	-	13.9

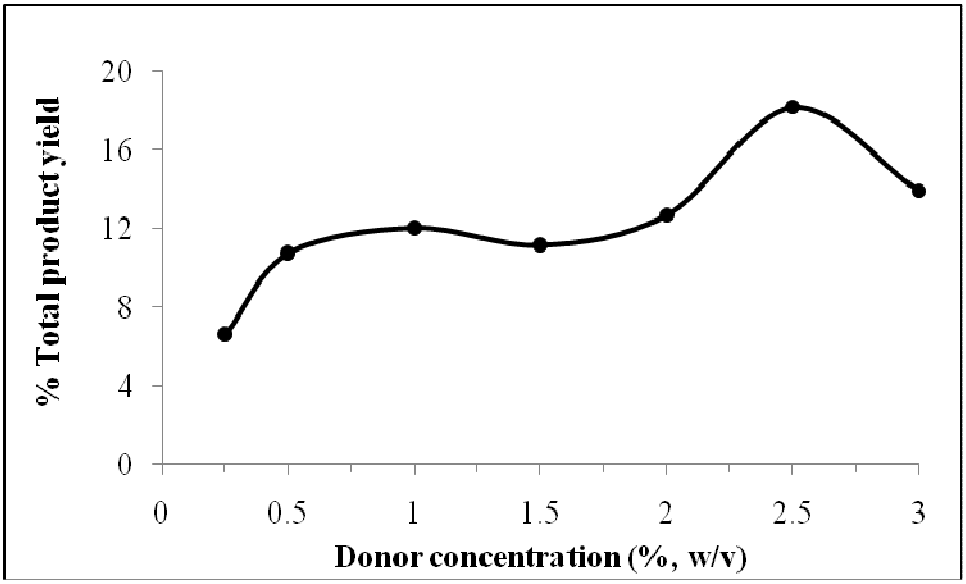


Figure 3.12 Effect of glucosyl concentration on amylomaltase transglycosylation yield

3.3.2.3 Effect of enzyme concentration

Using the condition as mentioned in section 3.3.2.2 with various concentrations of amylomaltase, it was found that product yields varied with amylomaltase concentration. The percent yield of each transfer product and total product yields are shown in Table 3.11 and Figure 3.13. The total product yield increased when amylomaltase concentration increased to 0.25-12 U/ml. A new product was observed at Rt 16 when amylomaltase concentration of 0.5-12 U/ml was used. The maximum product yield was around 63.5-63.7 % when the reaction mixture was incubated with 9-12 U/ml of amylomaltase. Therefore, the optimum amylomaltase concentration of 9 U/ml was used.

3.3.2.4 Effect of incubation time

Using the condition as mentioned in section 3.3.2.3 with various incubation times, it was found that product yields were clearly affected. The percent yield of each transfer product and total product yields are shown in Table 3.12 and Figure 3.14. The total product yield at 6 hours incubation was rather low when compared with 12 hours. The maximum product yield was obtained around 81.7-82.0% when incubation time was 48-72 hours. To save on time, the 48 hours was chosen for the optimum incubation time.

Table 3.11 Percent yields of transglycosylated products at different amylomaltase concentrations

Amylomaltase concentration (U/ml)	Product yield (%)										Total yield (%)
	Rt 16	Rt 17	Rt 18	Rt 19	Rt 20	Rt 22	Rt 24	Rt 26	Rt 30	Rt 34	
0.25	-	0.19	0.39	0.61	0.72	0.79	0.78	0.74	0.65	-	4.85
0.5	0.20	0.33	0.67	1.03	1.23	1.39	1.40	1.33	1.17	0.43	9.17
0.75	1.60	1.85	1.97	2.09	2.14	2.07	1.91	1.86	1.66	0.69	17.9
1	1.82	2.13	2.26	2.38	2.45	2.41	2.33	2.24	2.00	0.95	21.0
3	3.11	3.22	3.60	4.01	4.42	4.68	4.84	4.98	4.81	2.82	40.5
5	2.77	3.58	4.21	4.86	5.54	6.13	6.48	6.63	6.57	4.73	53.5
7	2.97	2.96	3.78	4.76	5.49	6.86	7.79	8.58	9.02	8.84	61.0
9	2.70	1.99	2.94	3.94	5.29	6.74	7.41	9.01	11.7	11.8	63.5
12	2.72	2.40	1.74	2.69	4.18	6.35	7.56	11.6	12.1	12.2	63.7

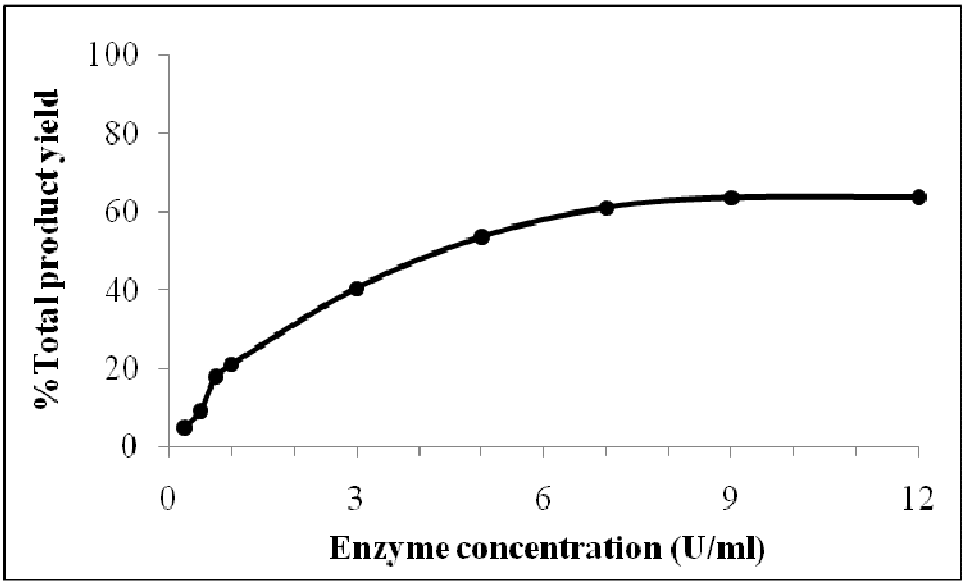


Figure 3.13 Effect of enzyme concentration on amylomaltase transglycosylation yield

Table 3.12 Percent yields of transglycosylated products at different incubation time with amylomaltase

Incubation time (hour)	Product yield (%)										Total yield (%)
	Rt 16	Rt 17	Rt 18	Rt 19	Rt 20	Rt 22	Rt 24	Rt 26	Rt 30	Rt 34	
6	3.63	3.79	3.63	3.90	4.17	4.36	4.48	4.39	4.04	2.78	39.2
12	3.96	4.38	4.53	5.19	5.92	6.65	7.20	7.41	7.35	5.90	58.5
18	3.02	3.32	3.78	4.77	5.99	7.39	8.88	10.4	11.4	10.4	69.3
24	3.53	3.88	4.35	5.29	6.47	7.72	9.03	10.3	11.1	10.1	71.7
36	3.33	4.12	4.56	5.21	6.50	8.02	9.93	11.6	12.9	12.4	78.6
48	3.20	3.86	5.45	5.46	6.20	7.81	9.81	11.9	13.9	14.0	81.7
72	3.43	3.23	4.90	5.7	5.61	7.84	9.89	12.6	14.4	14.4	82.0

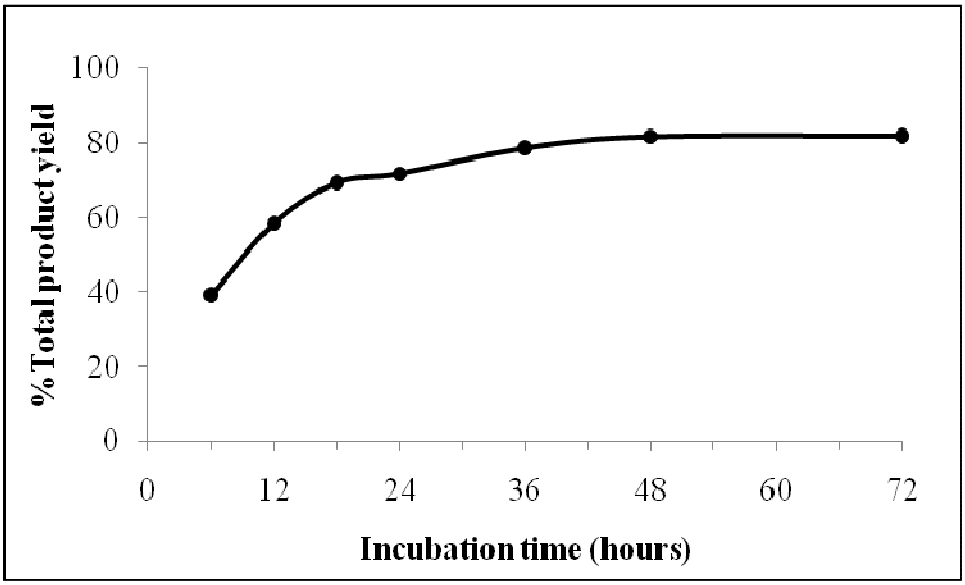
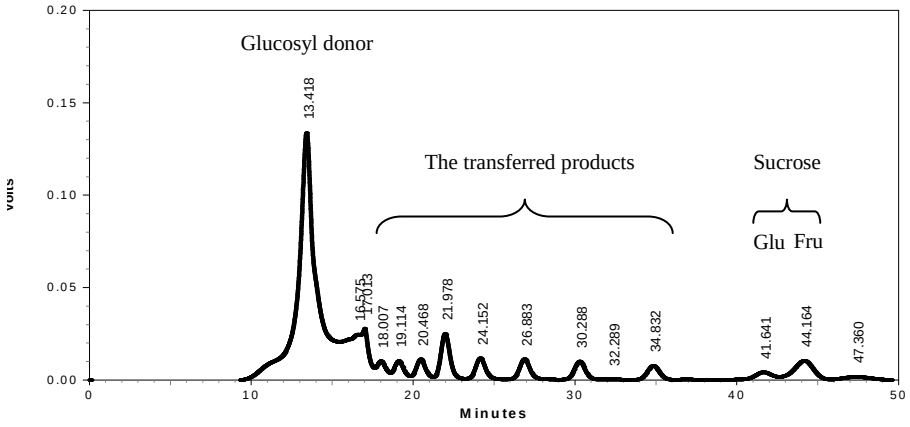


Figure 3.14 Effect of incubation time on amylomaltase transglycosylation yield

From the overall result, the optimum condition for transglycosylation to sucrose by CGTase from *Paenibacillus sp*. RB01 was: to incubate 400 U/ml of CGTase with 20% (w/v) soluble tapioca starch and 2.5% (w/v) sucrose at 40°C for 18 hours. The total transglycosylation yield was 98.6% and at least 7 products were found at Rt 18 (9.11%), Rt 19 (11.7%), Rt 20 (11.9%), Rt 24 (17.3%), Rt 26 (18.2%), Rt 30 (16.3%) and Rt 34 (14.1%), respectively. The profile of reaction products after optimization was shown in Figure 3.15 and compared with Figure 3.4 that before optimization.

The optimum condition for transglycosylation to sucrose by amylomaltase from recombinant amylomaltase was: to incubate of 9 U/ml of amylomaltase with 2.5% (w/v) raw tapioca starch and 2.0% (w/v) sucrose at 30°C for 48 hours. The total transglycosylation yield was 81.7%. Although transglycosylation reaction with CGTase gave at least 7 products, for amylomaltase at least 10 products were formed at Rt 16 (3.20%), Rt 17 (3.86%), Rt 18 (5.45%), Rt 19 (5.46%), Rt 20 (6.20%), Rt 22 (7.81%), Rt 24 (9.81%), Rt 26 (11.9%), Rt 30 (13.9%) and Rt 34 (14.0%), respectively. The profile of reaction products after optimization was shown in Figure 3.15 and compared with Figure 3.10 that before optimization. Only the transfer products of amylomaltase was further characterized and determined for their biological properties because this is the first work to report the use of amylomaltase in the synthesis of maltosylsucrose and to characterize its transferred products.

A)



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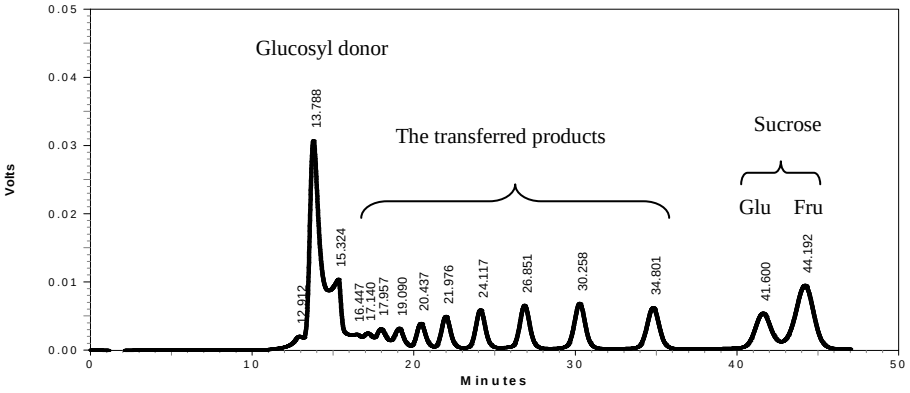


Figure 3.15 HPLC chromatogram of reaction products after optimization

A) The transfer products of CGTase

B) The transfer products of amylomaltase

3.4 Larger scale preparation and isolation of maltosylsucrose products

The larger scale of reaction mixture (25 ml) was prepared as described in section 2.13 using optimum condition for transglycosylation obtained in section 3.3. After transglycosylation reaction, the reaction mixture was centrifuged for 30 minutes at 10,000 x g and then applied on Biogel-P2 column. The column was eluted with distilled water at a flow rate of 15 ml/hour. The fractions containing sugar were detected by phenol-sulfuric method. The Biogel-P2 column profile of reaction products from CGTase is shown in Figure 3.16, more than ten peaks (a to l) were observed. The Biogel-P2 column profile of reaction products from amylomaltase was shown in Figure 3.18. About ten peaks (A to J) were also observed and they were identified by HPLC as described in the section 2.10.2.2.

The HPLC chromatogram of the transglycosylated products formed by CGTase is shown in Figure 3.17. From preliminary result, glucosyl donors (soluble tapioca starch) were determined at Rt 13, 15, 16, 17 min. Therefore, Peak a, b and c were glucosyl donors, Peak d to Peak k were composed of more than one products formed at Rt 18 to 34 min and Peak l was sucrose, which was hydrolyzed to glucose and fructose after exposure to high temperature (80°C) in the HPLC column at Rt 41 and Rt 44 min (Table 3.13).

From HPLC chromatogram of the products by amylomaltase (Figure 3.19), Peak A and B were glucosyl donor (raw tapioca starch) at Rt 13 and Rt 15 min, Peak C to I were composed of more than one product from Rt 16 to 34 min while Peak J was sucrose hydrolyzed to glucose and fructose at Rt 41 and Rt 44 (Table 3.14). Thus, Peak C to I were concentrated with a Centrivap Concentrator. The concentrated products were further characterized for size by Mass spectrometry.

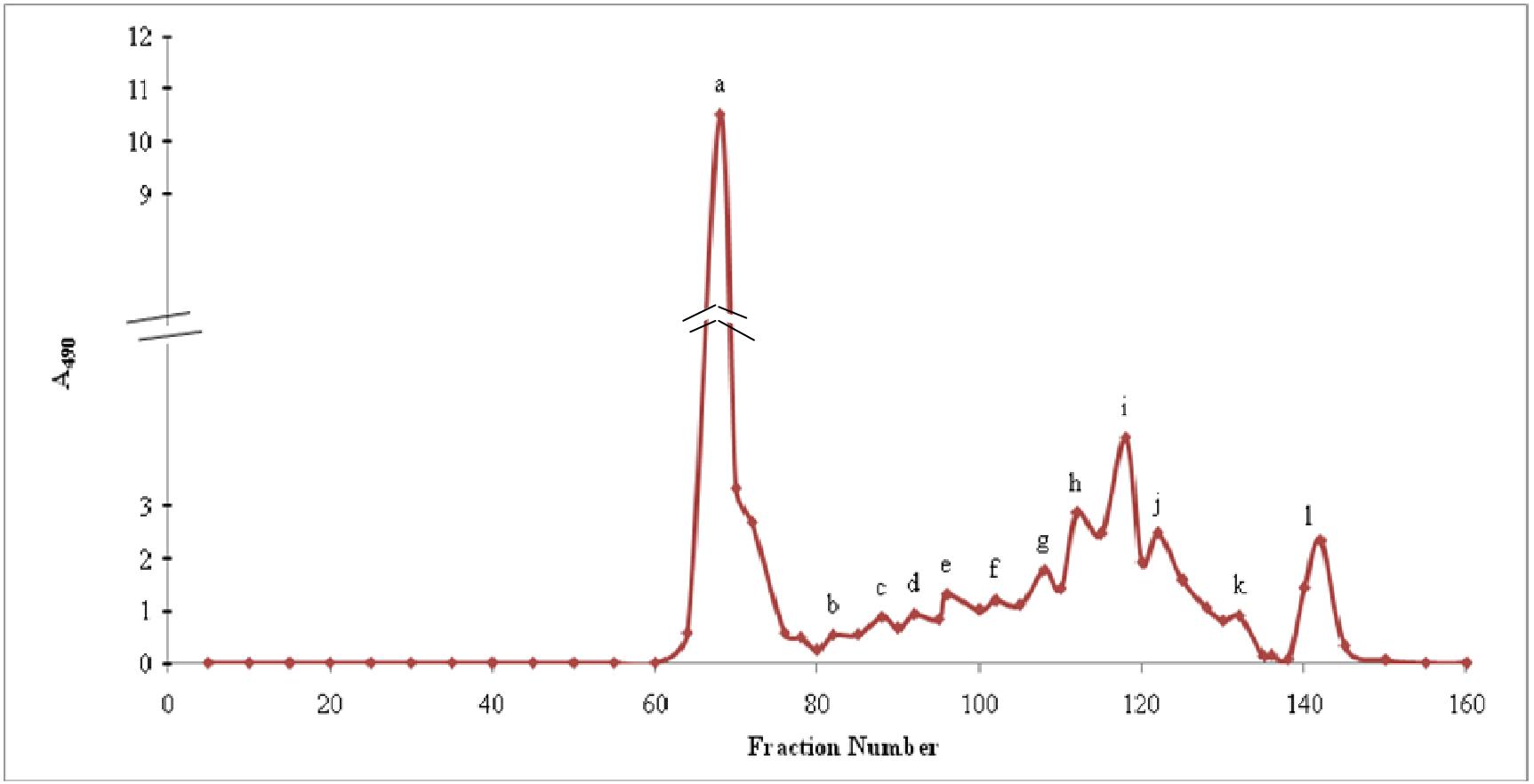
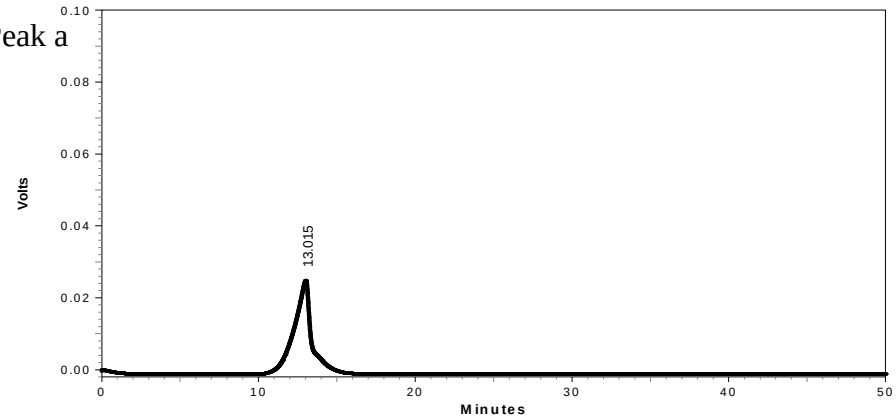
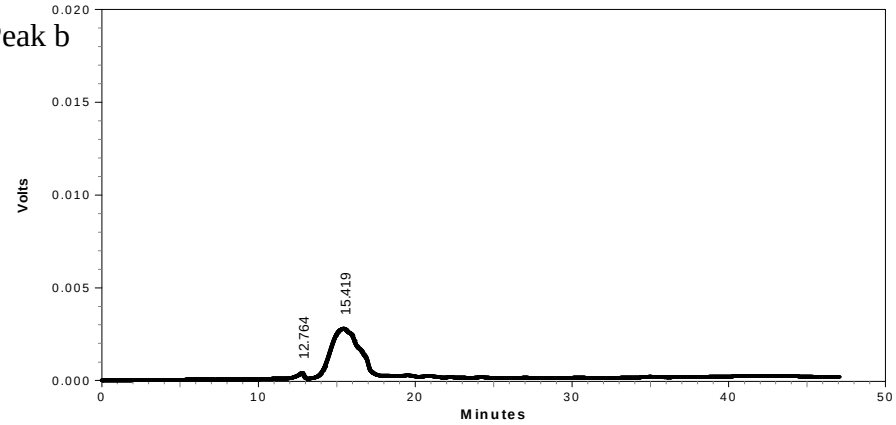


Figure 3.16 Biogel-P2 column profile of reaction products by CGTase, column size 1.80x80 cm, flow rate 15 ml/hour, fraction size 1 ml, distilled water as eluent

A) Peak a



B) Peak b



C) Peak c

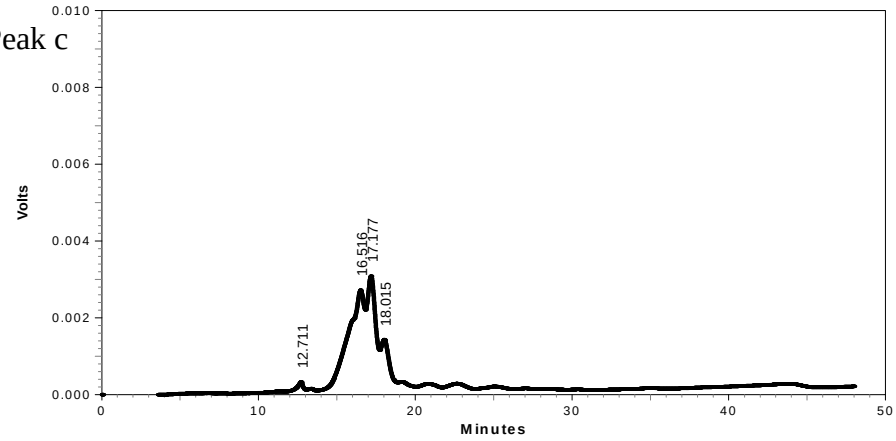
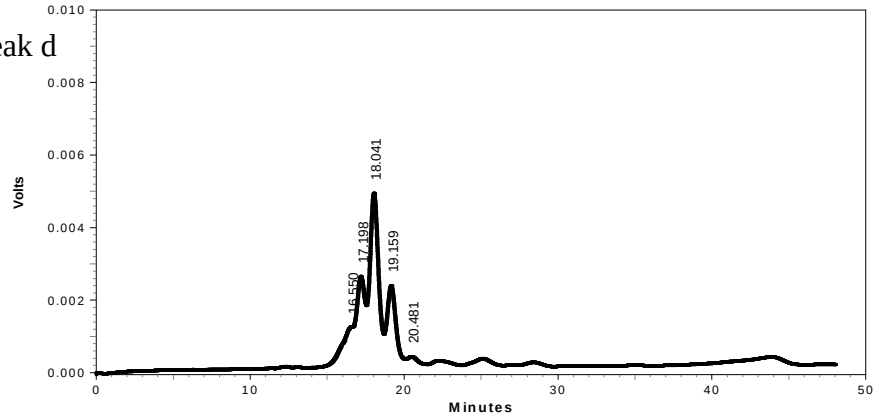
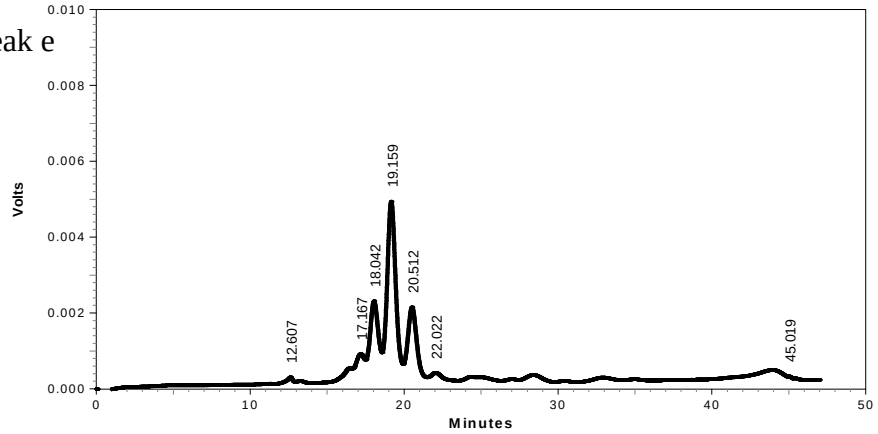


Figure 3.17 HPLC chromatogram of peaks a to l collected from Biogel-P2 column

D) Peak d



E) Peak e



F) Peak f

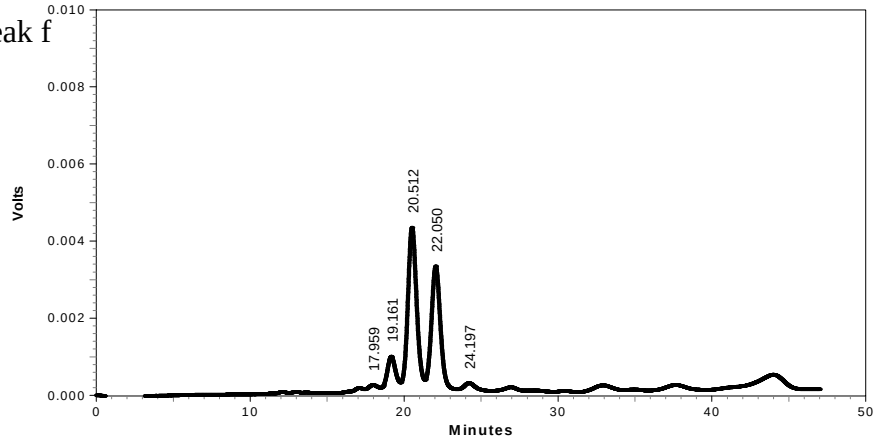
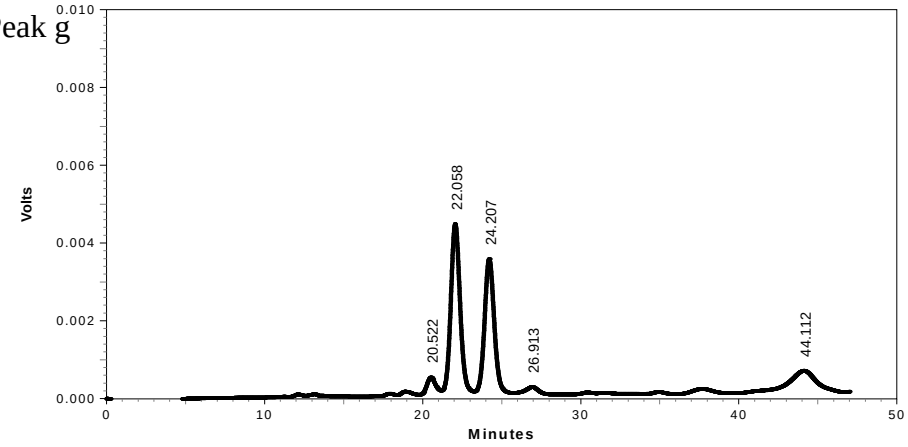
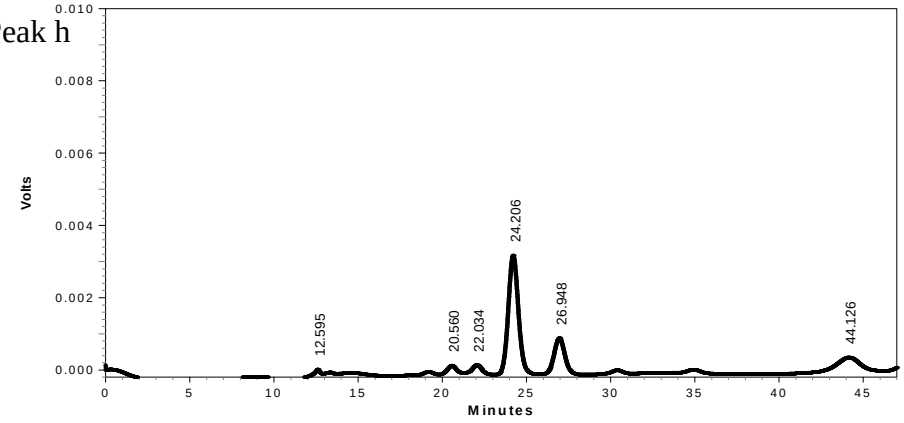


Figure 3.17 (continue) HPLC chromatogram of peaks a to l collected from Biogel-P2 column

G) Peak g



H) Peak h



I) Peak i

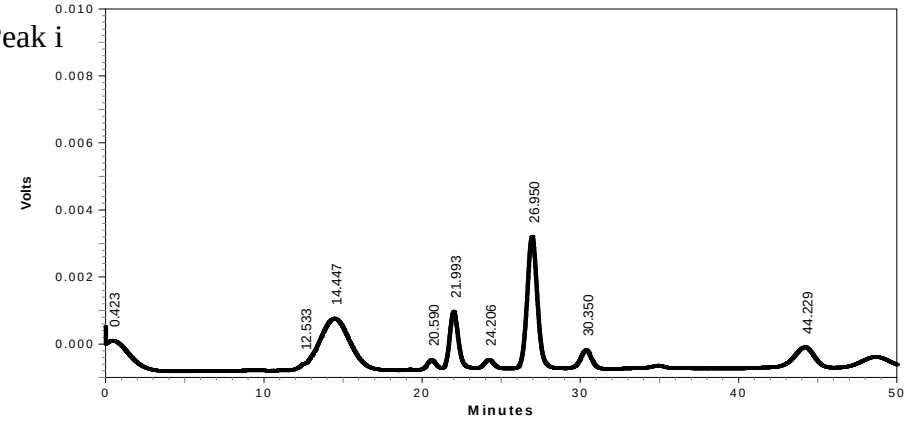
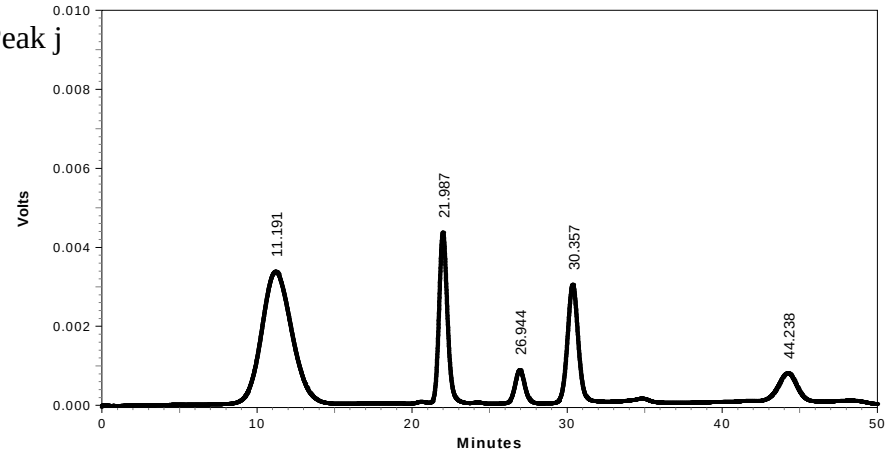
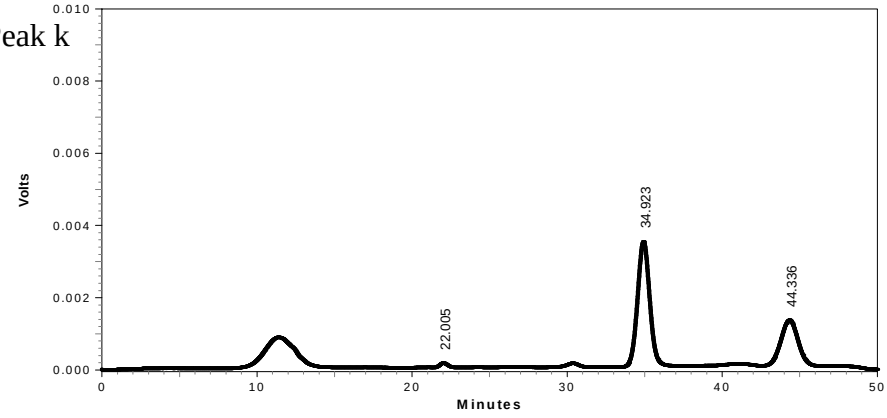


Figure 3.17 (continue) HPLC chromatogram of peaks a to l collected from Biogel-P2 column

J) Peak j



K) Peak k



L) Peak l

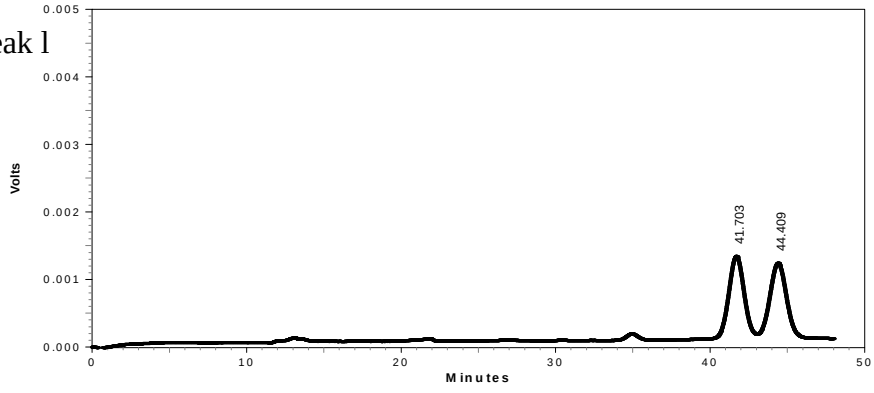


Figure 3.17 (continue) HPLC chromatogram of peaks a to l collected from Biogel-P2 column

Table 3.13 Identification of Peak a to l collected from Biogel-P2

Peak	Rt (min)
a	13
b	13, 15
c	13, 16, 17, 18
d	16, 17, 18, 19, 20
e	13, 17, 18, 19, 20, 22, 44
f	18, 19, 20, 22, 24
g	20, 22, 24, 26, 44
h	13, 20, 22, 24, 26,44
i	15, 20, 22, 24, 26, 30, 44
j	11, 22, 26, 30, 44
k	22, 34, 44
l	41*,44**

* Rt 41 - glucose

** Rt 44 - fructose

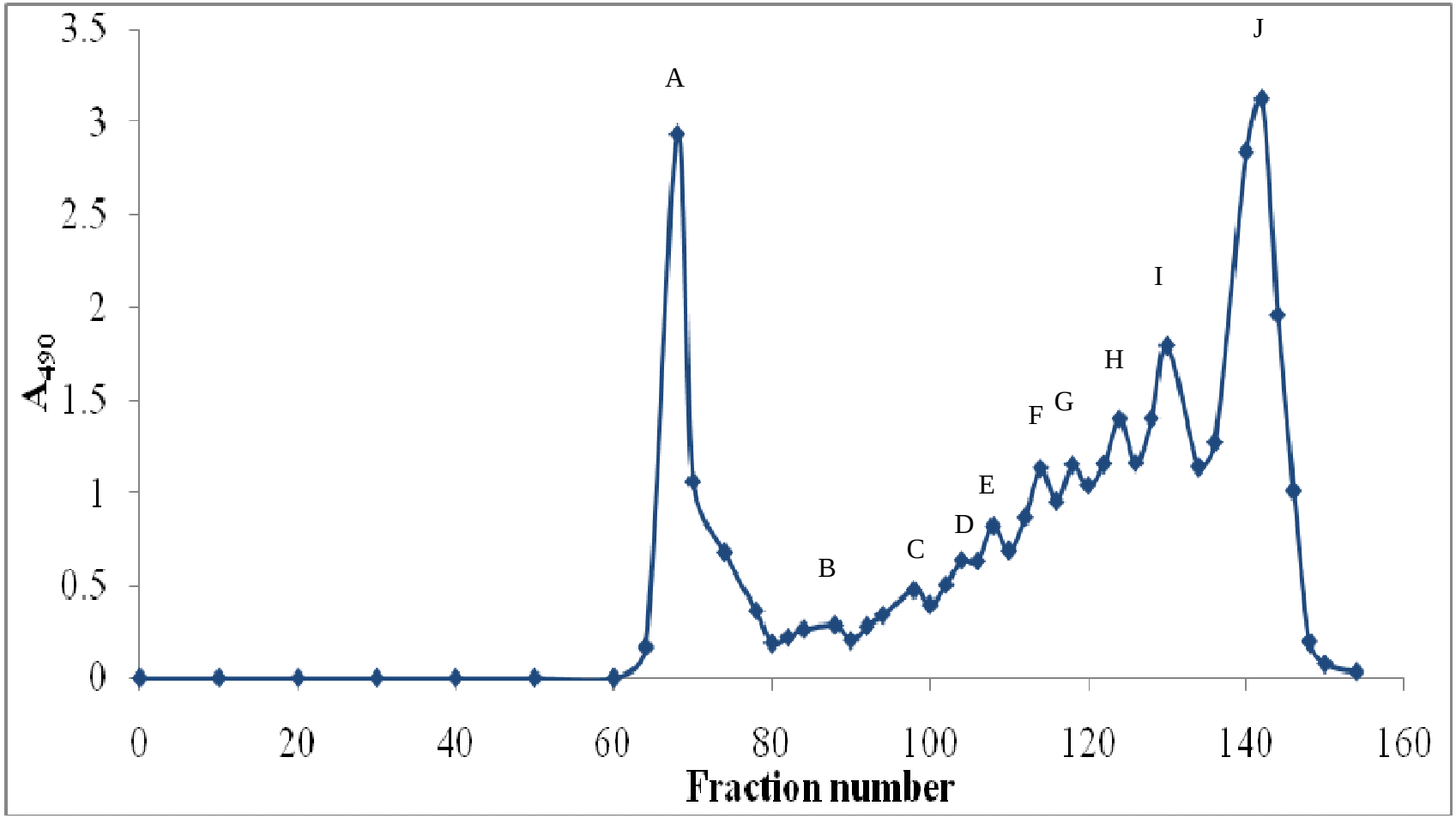
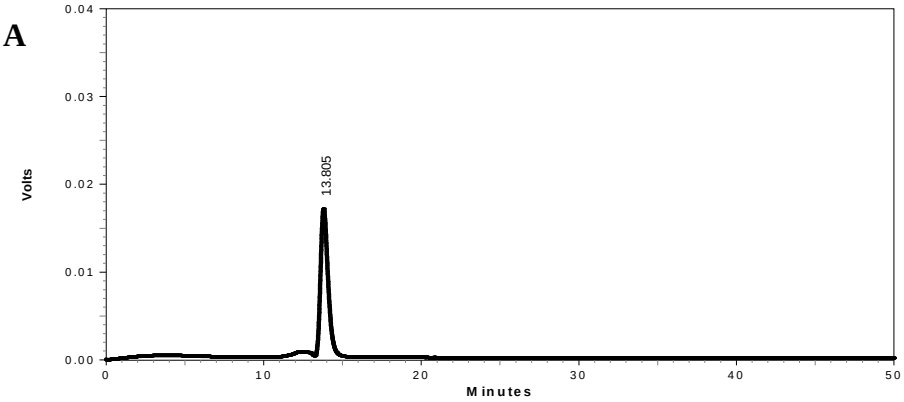
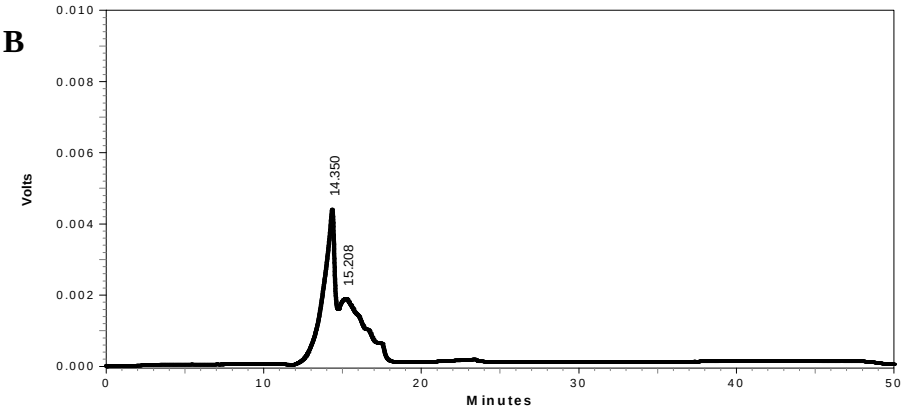


Figure 3.18 Biogel-P2 column profile of reaction products by amylomaltase, column size 1.80x80 cm, flow rate 15 ml/hour, fraction size 1 ml, distilled water as eluent

A) peak A



B) peak B



C) peak C

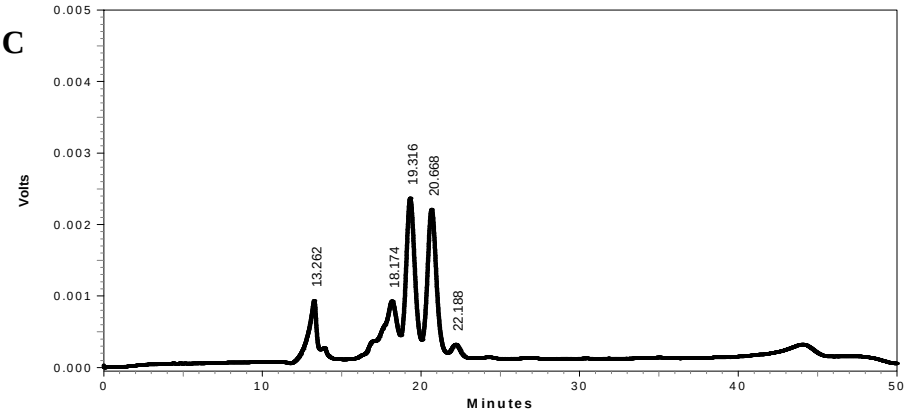
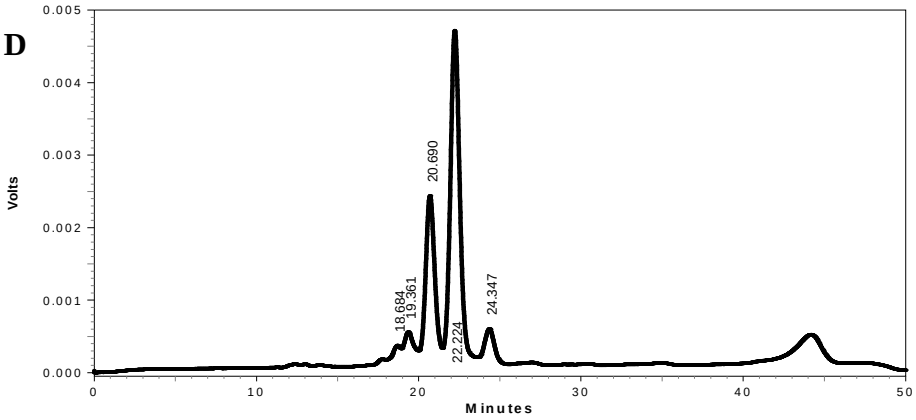
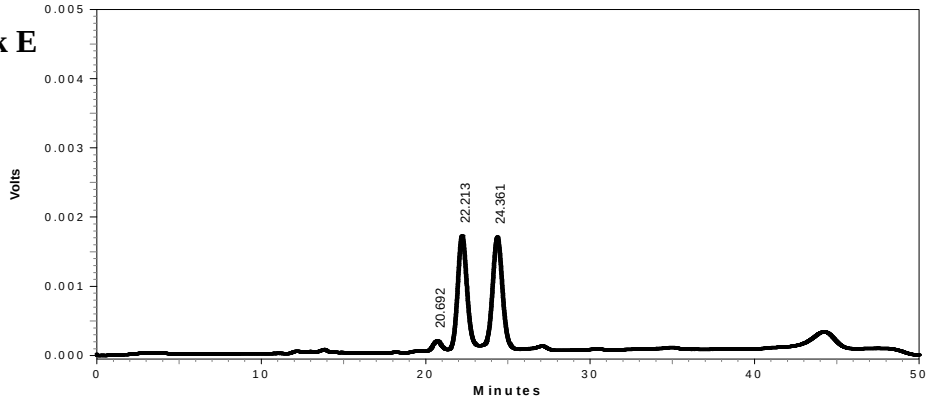


Figure 3.19 HPLC chromatogram of peaks A to J collected from Biogel-P2 column

D) peak D



E) peak E



F) peak F

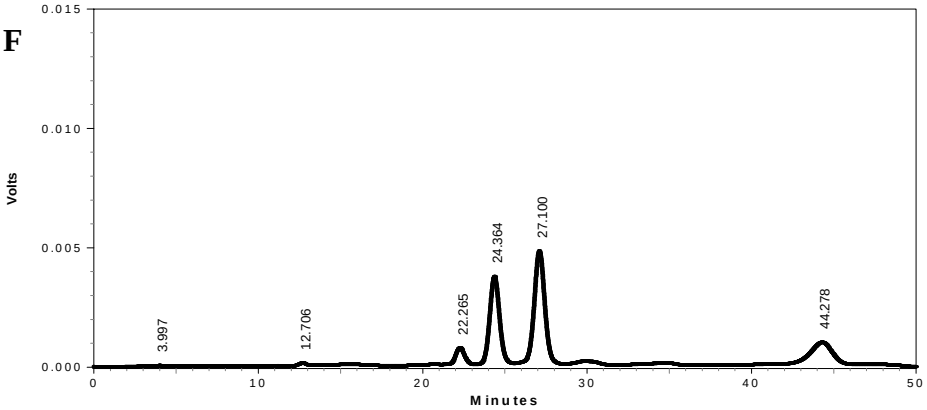
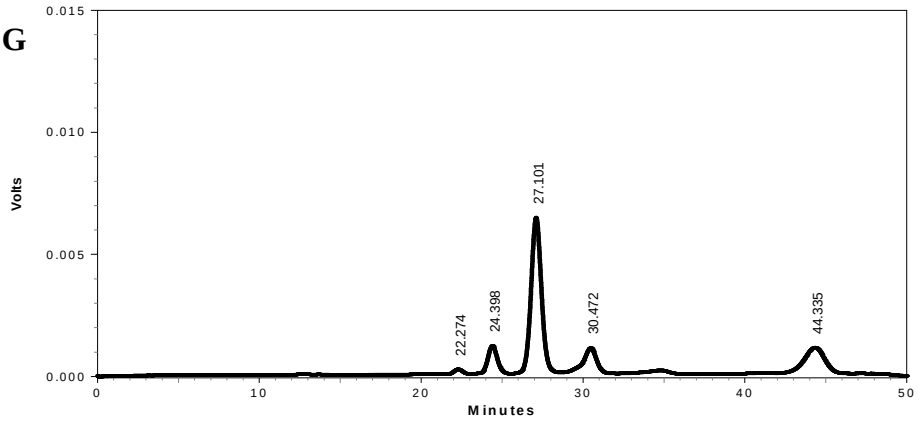
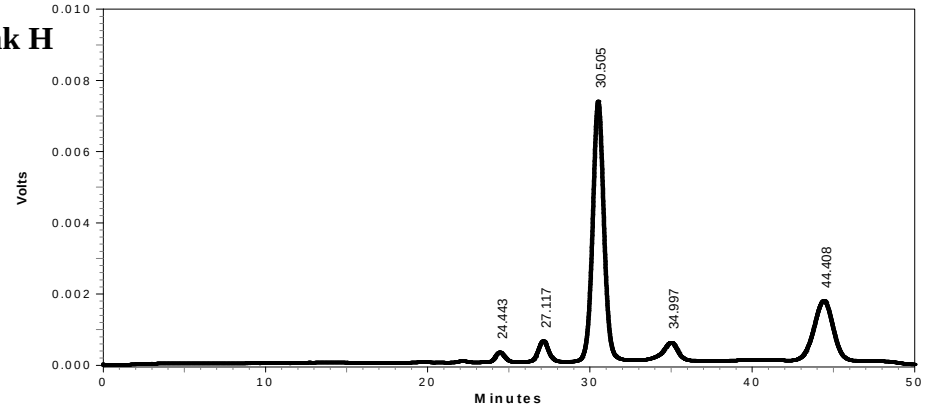


Figure 3.19 (continue) HPLC chromatogram of peaks A to J collected from Biogel-P2 column

G) peak G



H) peak H



I) peak I

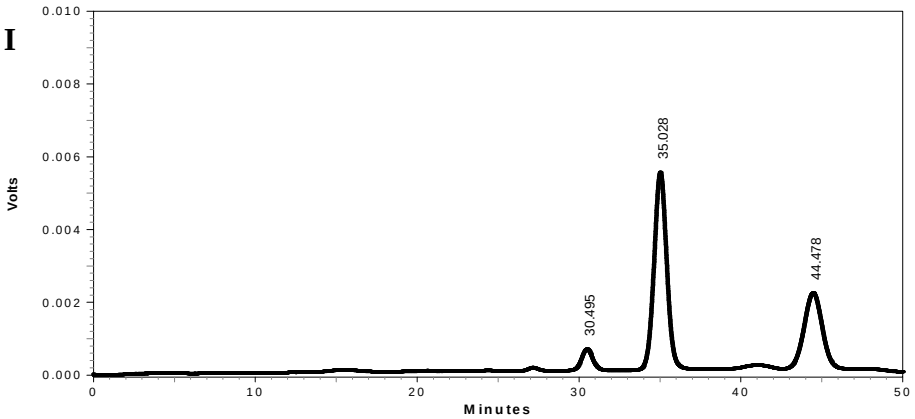


Figure 3.19 (continue) HPLC chromatogram of peaks A to J collected from Biogel-P2 column

J) peak J

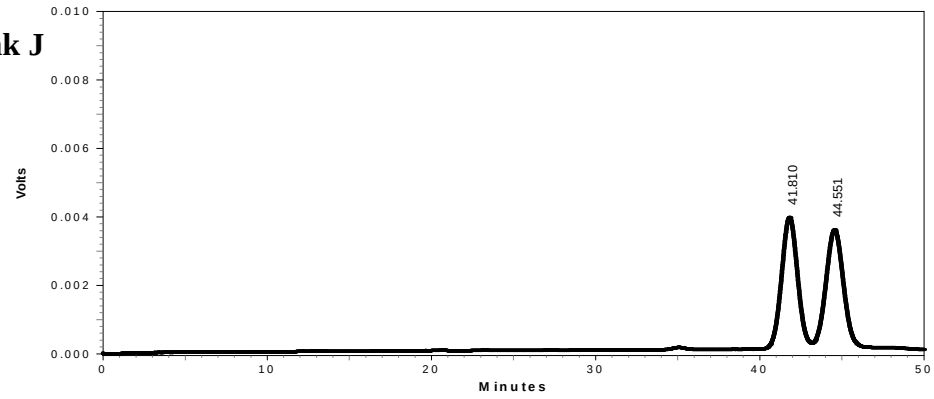


Figure 3.19 (continue) HPLC chromatogram of peaks A to J collected from Biogel-P2 column

Table 3.14 Identification of Peak A to J collected from Biogel-P2

Peak	Rt (min)
A	13
B	14, 15
C	13, 18, 19, 20, 22
D	18, 19, 20, 22, 24
E	20, 22, 24
F	22, 24, 26, 44
G	24, 26, 30, 44
H	24, 26, 30, 34, 44
I	30, 34, 44
J	41*, 44**

* Rt 41 - glucose

** Rt 44 - fructose

3.5 Characterization of Products

3.5.1 Mass Spectrometry

The molecular weights of the synthesized products by amylomaltase were elucidated by mass spectrometry as described in section 2.13. The molecular weight of the product at Rt 34 was estimated to be 504 daltons (by ESI-ToF mass spectrometry) with [M+Na]+ at m/z of 527 (Figure 3.20) and this corresponded to the size of trisaccharide of maltosyl fructoside (G_2F).

The product at Rt 30 min, the molecular weight was estimated to be 666 daltons with [M+Na]+ at m/z of 689, this corresponded to the size of tetrasaccharide of maltotriosyl fructoside (G_3F) and the product at Rt 26 min , the molecular weight was 828 daltons with [M+Na]+ at m/z of 851, this corresponded to the size of pentasaccharide of maltotetraosyl fructoside (G_4F) (Figure 3.21).

The product at Rt 24 min, the molecular weight was estimated to be 990 daltons with [M+Na]+ at m/z of 1013 (Figure 3.22), this corresponded to the size of hexasaccharide of maltopentaosyl fructoside (G_5F). The product at Rt 22 min, the molecular weight was 1152 daltons with [M+Na]+ at m/z of 1175, this corresponded to the size of heptasaccharide of maltohexaosyl fructoside (G_6F) and the product at Rt 20 min, the molecular weight was 1314 daltons with [M+Na]+ at m/z of 1337, this corresponded to the size of octasaccharide of maltoheptaosyl fructoside (G_7F) (Figure 3.22).

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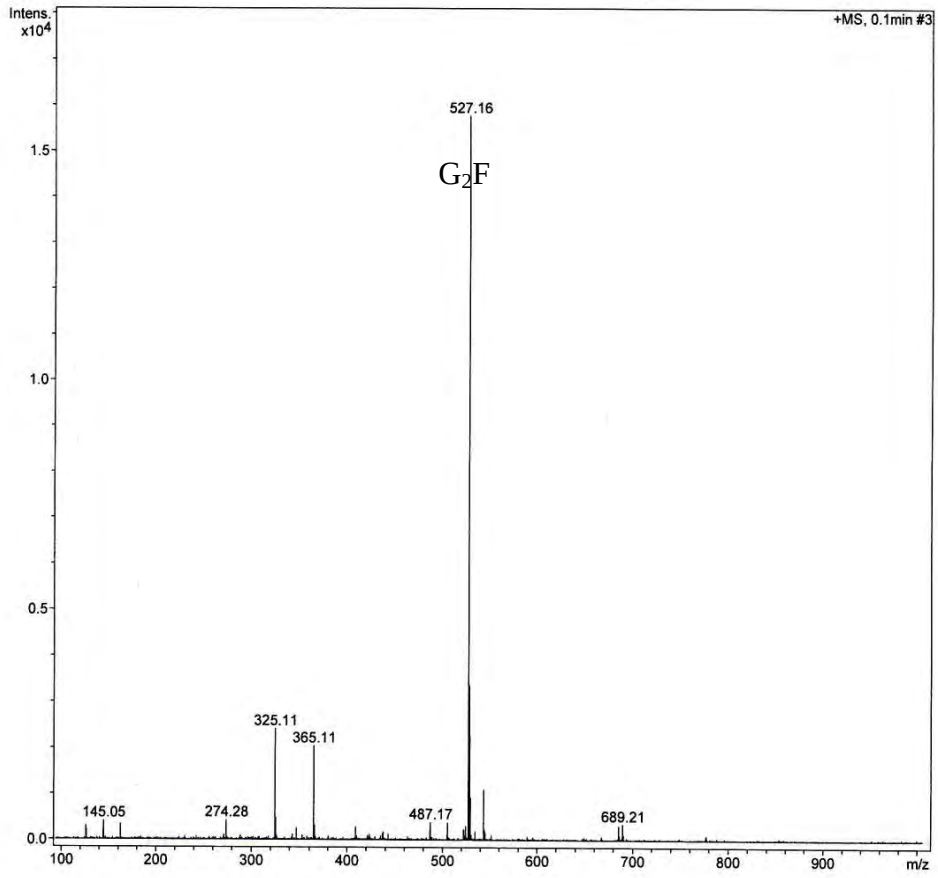

Figure 3.20 ESI-TOF mass spectrum of the product at Rt 34 from Peak I

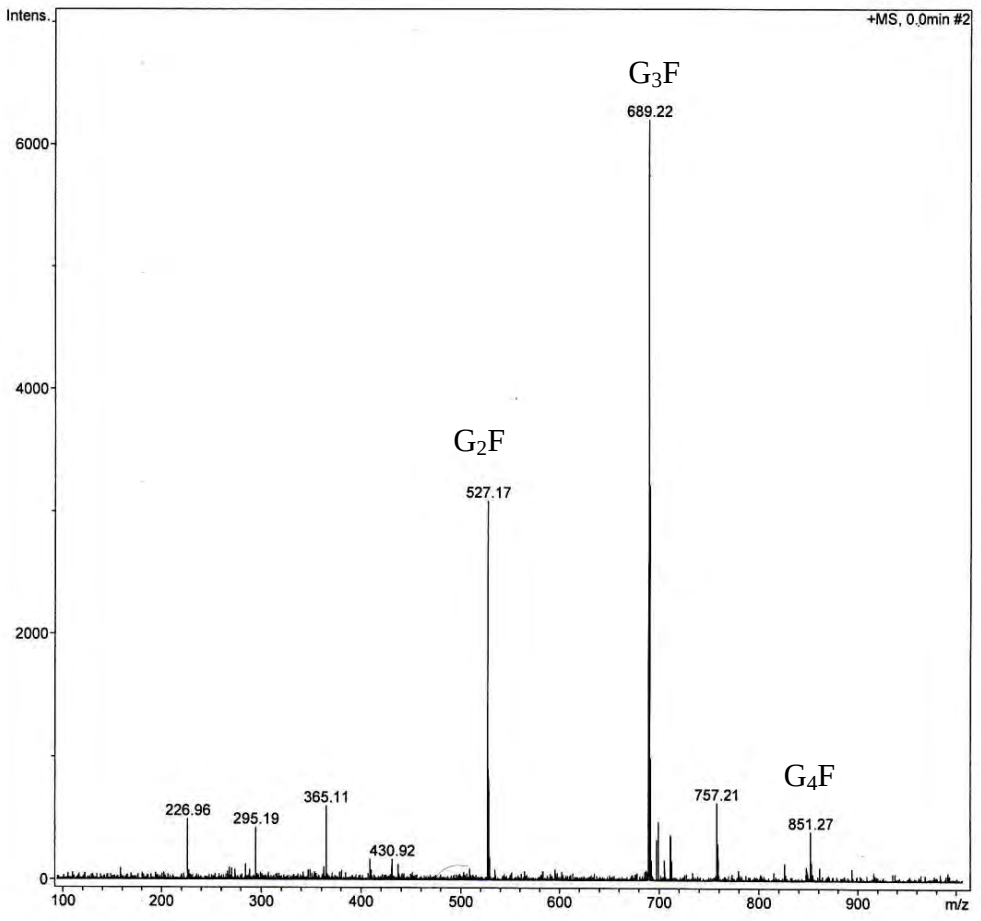

Figure 3.21 ESI-TOF mass spectrum of the product at Rt 26, 30 and 34 from Peak H

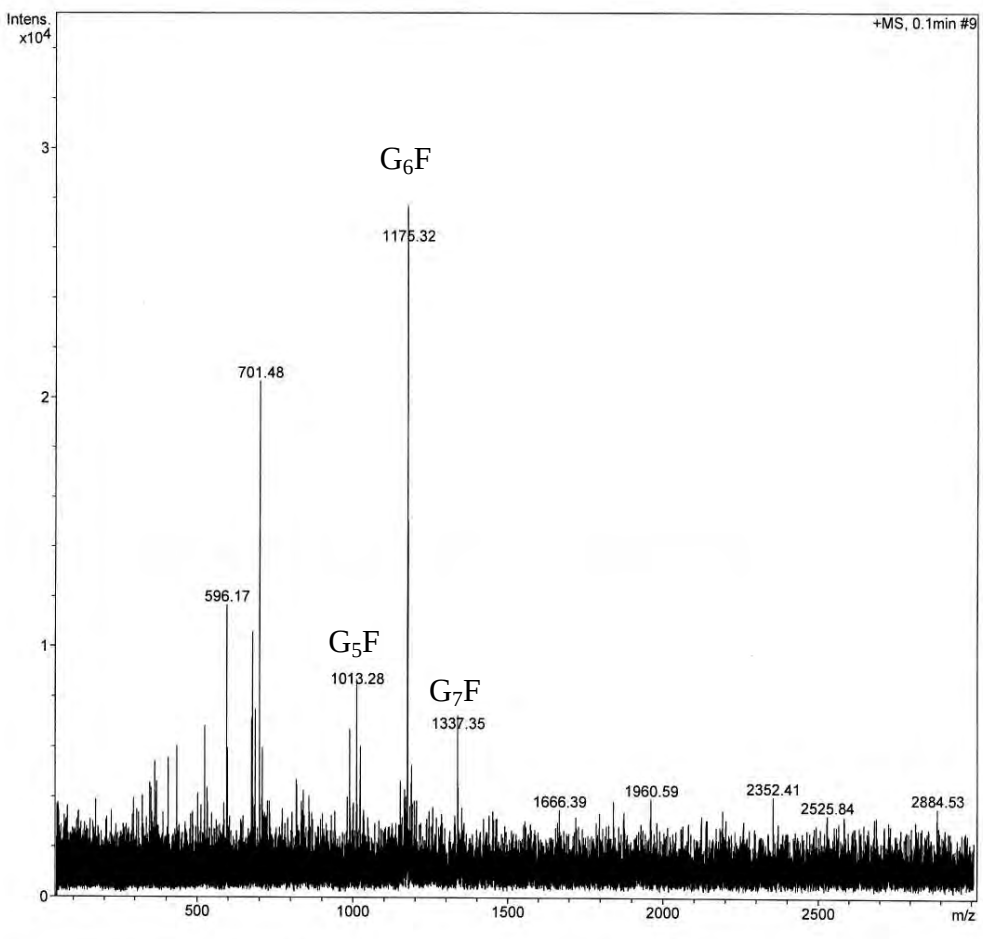

Figure 3.22 ESI-TOF mass spectrum of the product at Rt 20, 22 and 24 from Peak D

3.6 Determination of biological property of products

3.6.1 The inhibitory effects of maltosylsucrose on the synthesis of water insoluble glucan

When 40 mg of sucrose, the mixture of maltosylsucrose or commercial coupling sugar were incubated with 25 µl pre-cultured *S. mutans* at 37°C for 24 h, the amount of insoluble glucan produced by GTase from *S. mutans* was reduced by about 16% for the mixture of maltosylsucrose and about 19% for commercial coupling sugar compared to that formed by sucrose alone (Table 3.15). The results suggested that the mixture of maltosylsucrose and commercial coupling sugar could lead to the synthesis of a water insoluble glucan, but the amount was less than that exerted by sucrose.

3.6.2 The inhibitory effects of maltosylsucrose on the acid fermentation

When 17 mg of all three sugars tested were supplied to the test system of acid fermentation and incubated at 37°C for 60 hours, the pH of the reaction mixture dropped from pH 7.30 to pH 4.37, 5.70 and 5.62 when incubated with sucrose, the mixture of maltosylsucrose and commercial coupling sugar, respectively (Table 3.15). These results suggested that the mixture of maltosylsucrose and commercial coupling sugar could induce fermentation, but less than that by sucrose.

3.6.3 The inhibitory effects of maltosylsucrose on the plaque formation

When 50 mg of all three sugars tested were incubated with 25 µl pre-cultured *S. mutans* at 37°C for 24 hours, the amount of adherent plague on test tube surfaces were 59.5 mg, 34.0 mg and 28.0 mg when incubated with sucrose, the mixture of maltosylsucrose and commercial coupling sugar, respectively (Table 3.15). These results suggested that the mixture of maltosylsucrose and commercial coupling sugar also inhibited the plaque formation on glass surface.

3.6.4 The inhibitory effects of maltosylsucrose on cell aggregation

When 0.2 mg of all three sugars tested were incubated with *S. mutans* cells at 37°C for 18 hours, sucrose induced aggregation of cells more than the mixture of maltosylsucrose and commercial coupling sugar with 4+, 2+ and 2+ score, respectively (Table 3.15). The results indicated that the mixture of maltosylsucrose and commercial coupling sugar reduced cell aggregation as compared to sucrose.

3.6.5 The inhibitory effects of maltosylsucrose on GTase activity

The inhibitory effects of the mixture maltosylsucrose and commercial coupling sugar on GTase activity in the presence of sucrose is shown in Table 3.16 and Figure 3.23. When the ratio of the amount of the mixture of maltosylsucrose and coupling sugar to sucrose was 1:1, the enzyme activity of *S. mutans* was inhibited by 7.1 and 10.5%, respectively. With the ratio of the maltosylsucrose mixture and coupling sugar to sucrose of 2:1, the GTase activity of *S. mutans* was inhibited by 33.2 and 40.0%, respectively, and when the ratio increased to 4:1 the GTase activity was inhibited by 50.7 and 58.6%, respectively. These results suggested that the mixture of maltosylsucrose and commercial coupling sugar could result in inhibition of the activity of GTase exerted by sucrose substrate.

Table 3.15 Effect of sucrose, the mixture of maltosylsucrose and commercial coupling sugar as a substrate on inhibition of *S. mutans*

Substrate	Relative ratio of insoluble glucan synthesis[a]	pH after 60 h incubation	plague (mg)	Aggregation
Sucrose	1.00 ± 0.065	4.37 ± 0.01	59.5 ± 1.1	4+
The mixture of maltosylsucrose	0.83 ± 0.094	5.70 ± 0.01	34.0 ± 0.6	2+
Commercial coupling sugar	0.81 ± 0.028	5.62 ± 0.01	28.0 ± 1.7	2+

[a] Relative ratio of insoluble glucan synthesis was calculated by comparing the optical density at 550 nm. The optical density observed with sucrose was used as a control

Each value is mean ± SD from three separate experiments.

Table 3.16 Inhibition of GTase activity with sucrose substrate by the mixture of maltosylsucrose and coupling sugar

Ratio of substrate		Insoluble glucan synthesis (µmole/min, x 10^{-2})	Relative activity (%)
Sucrose: Maltosylsucrose mixture	1:0	2.80	100
	1:1	2.60	92.9
	1:2	1.87	66.8
	1:4	1.38	49.3
Sucrose:Coupling sugar	1:0	2.85	100
	1:1	2.55	89.5
	1:2	1.71	60.0
	1:4	1.18	41.4

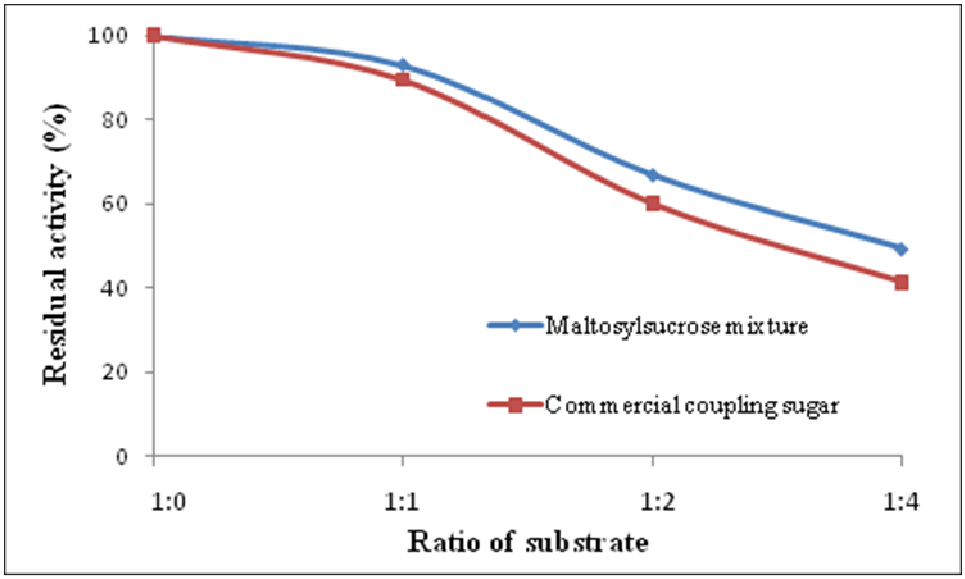

Figure 3.23 The inhibitory effect of the mixture of maltosylsucrose and commercial coupling sugar on GTase activity in the presence of sucrose

CHAPTER IV

DISCUSSION

4.1 Purification of CGTase

CGTase used in this study was from *Paenibacillus* sp. RB01, the thermotolerant bacteria isolated from hot spring area in Ratchaburi province, Thailand (Tesana, 2001). The crude extracellular CGTase was collected by centrifugation and then partially purified by starch adsorption method (Kato and Horikoshi, 1985, cited in Laloknam, 1997). CGTase activity was determined by the starch degrading (dextrinizing) activity assay, the degrading of soluble potato starch was measured with the decrease of starch-iodine complex (Fuwa, 1954, with slight modification by Techaiyakul, 1991). The enzyme was partially purified to 22.9 fold with 56.1% yield (Table 3.1). Previous report on partial purification of this enzyme by the same method gave 69.8% yield with 14.6 fold increase in purity (Aramsangtienchai, 2007). From SDS-PAGE (Figure 3.3), the CGTase band of ≈ 66 kDa (Yenpetch, 2000) was approximately 3/4 of total protein and only one faint protein band of lower molecular weight at around 47 kDa was contaminated. Thus, the purity of this partially purified enzyme was high enough for using in further transglycosylation study.

4.2 Purification of amylomaltase

The recombinant amylomaltase (pCGAM), was produced by *Escherichia coli* BL21 (DE3) with pET-19b vector harboring amylomaltase gene from *Corynebacterium glutamicum* ATCC 13032. The crude enzyme was obtained by sonication of the cells and then the supernatant was collected by centrifugation. pCGAM was efficiently purified by a prepacked HisTrap affinity column, which is a metal-affinity chromatography using precharged Ni Sepharose™ High Performance (Amersham Biosciences, 2004). A histidine-tagged recombinant protein with a stretch of 6 histidine residues (His-tag) linked to the N- or C-terminal part of the recombinant

protein is sufficient for a high affinity interaction with the Ni^{2+} (Schmitt *et al*, 1993). pCGAM contained 10 histidine residues, it was thus strongly bound to the column. The bound proteins are then eluted from the resin upon protonation of the interacting amino acid side chains or by displacement with other metal binding ligands such as imidazole, a precursor of histidine (Schmitt *et al*, 1993). pCGAM was eluted from the column by imidazole solution. Amylomaltase activity was determined from the disproportionation reaction of soluble potato starch and maltose acceptor by the iodine method (Srisimarat, 2010). The recombinant amylomaltase was purified to 15.4 fold with 47.3% yield (Table 3.1). The previous work by Srisimarat (2010) reported a 30.2% yield with 10.8 fold increase in purity. From SDS-PAGE (Figure 3.3), the amylomaltase band of ≈ 84 kDa was about 3/4 of total protein and only one very faint protein band of lower molecular weight at around 72 kDa was contaminated. Thus, the purity of this purified enzyme was high enough for using in further transglycosylation study.

4.3 Synthesis of maltosylsucrose by CGTase and detection of the products

The synthesis of maltosylsucrose from the transglycosylation activity of CGTase using sucrose acceptor and various glucosyl donors was performed and the products were analyzed by TLC. This chromatographic separation is based on the distribution of compound between two phases, a mobile phase and a stationary phase. In this study, silica gel is used to be the stationary phase, and the migration of compounds on stationary phase depends on the polarity of substances (Sherma and Fried, 1991). Sucrose acceptor is a weak polar compound and weakly adsorbed with silica gel, thus it moves far from the origin. While some of the glucosyl donors such as soluble starch from potato and tapioca are highly polar compound so they are strongly adsorbed with silica gel and located at origin. For maltoheptaose and β-CD which were also used as glucosyl donors, they are less polar than the starch that they migrate better than starch. While the maltosylsucrose products are more polar than sucrose but less polar than the glucosyl donors, so their migrations in the non-polar mobile phase (n-butanol/pyridine/water 5:4:1, v/v) were in between these two compounds.

From TLC results, we found that soluble starch from potato and tapioca, maltoheptaose and β-CD were all able to act as glucosyl donor for the transglycosylation of CGTase in a similar extent. All of the reaction mixture from different glucosyl donors gave the same four major transglycosylation products with similar amounts observable from the intensity of product spots (Figure 3.3). The migrations of these products were comparable with the standard G3 to G7. This suggests that the size of the main products were from trisaccharide to heptasaccharide. Tapioca soluble starch was then chosen as the most suitable glucosyl donor for our further study due to its local availability and low cost. In the previous work, two major trisaccharide transfer products from the transglycosylation of *Bacillus stearothermophilus* maltogenic amylases using maltotriose and sucrose substrate were reported from TLC analysis (Lee *et al*., 2003). From another report using *Thermoanaerobacter sp*. CGTase, the main maltosylsucrose products were trisaccharide to pentasaccharide with small amounts of those with DP up to 7-8 observed on TLC and HPLC (Martín *et al*., 2004).

4.4 Optimization of transglycosylation reaction of CGTase

To obtain higher yield of maltosylsucrose, optimization of CGTase catalyzed transglycosylation reaction was carried out. Several parameters such as concentrations of acceptor, donor and enzyme including incubation time were varied. The reaction products were analyzed by HPLC according to their DP (retention time decreases with the increase in DP of maltosylsucrose) using a Rezex RSO-oligosaccharide column. This type of oligosaccharide column contains sulfonated styrene-divinylbenzene spheres in 4% cross-link resin including silver ionic form, using the ion exclusion mechanism by which ion-exchange resins are used for the fractionation of neutral and ionic species. Ionic compounds are rejected by the resin, thus they are eluted in the void volume of the column and for nonionic or weakly ionic substances penetrate into the pores of the resin that they partition between the liquid inside and outside of the resin particles (Papadoyannis and Samanidou, 2005), this column has high resolution for oligosaccharides up to 18 DP (Phenomenex, 2011). Prior to the optimization of transglycosylation reaction, the total transglycosylation yield of transfer products was

about 49.2% (Table 3.4). The yield of 98.6% was obtained after optimization (Table 3.7), thus the total transglycosylation yield was about two-fold increased. The total transglycosylation yield was higher than the 83% yield that was reported for CGTase from *Thermoanaerobacter sp*. (Martín *et al*., 2004) in which soluble potato starch was used as glucosyl donor and the major maltosylsucrose was G_2F which was about 60% yield. However, the amount of each oligosaccharide in the present study was determined on the assumption that at the same concentration, each exerts the same refractive index.

When, the optimization for sucrose concentration was performed, 0-10% sucrose were incubated with 5 % soluble tapioca starch glucosyl donor. At 0% sucrose, besides the donor substrate peak, the product at 22 min was observed (Table 3.4), this should be resulted from transglycosylation reaction from glucosyl donor by CGTase. In the presence of sucrose acceptor, the products were observed at Rt 19, 20, 24, 26, 30, 34 min. More types of products could be observed from analysis by HPLC than TLC. At low sucrose concentration of 0.025-0.1%, the peaks at Rt 19 and 20 min were main products but at high sucrose concentration of 1.0-10%, the main products were Rt 30 and 34 min. This suggested that when sucrose was excess, it was possible that the enzyme continued to transfer glucose units of larger products on to sucrose leading to the synthesis of smaller products (Monthieu *et al*., 2003). After that, the glucosyl donor concentrations were optimized. The number of product was increased from 6 to 7 products, the additional Rt 18 min was observed when glucosyl donor concentrations were 10-25% (Table 3.5). It might be possible that, at high concentration of glucosyl donor, the tranglycosylation reaction to yield large product was observed.

For the effect of enzyme concentration, the yields of products were increased from 48.9 to 98.7% when enzyme concentrations were increased from 6.25 to 400 U/ml (Table 3.6). And since the main products were observed at Rt 24, 26 and 30 min for all reactions, this suggested that the concentration of enzyme had an effect on the amount, not the type of the products. Furthermore, when the incubation time was varied, the total transglycosylation yields of products were increased from 87.6 to

98.6% when incubated at 6 to 18 hours, respectively. At longer than 18 hours, the product at Rt 18 min was increased, this indicated that the DP of products increased with increasing reaction time. After optimization, the product yield was in the order of Rt 26 ≈ Rt 24 ≈ Rt 30 > Rt 34 > Rt 20 ≈ Rt 19 > Rt 18 (later by MS, proved to be G_5F≈ G_4F≈ G_3F > G_2F > G_7F ≈ G_8F > G_9F). The product ratio of our CGTase was different from commercial product prepared from *Thermoanaerobacter* sp. CGTase. The largest component of the commercial maltosylsucrose (coupling sugar) (Appendix F) was the product at Rt 34 min and Rt 30, 26, 24 and 22 min were in decreasing order.

4.5 Synthesis of maltosylsucrose by amylomaltase and detection of the products

In this study, we found that amylomaltase was also able to transfer the glucosyl moiety of glucosyl donors which are all α-glucan to sucrose molecule as analyzed by TLC (Figure 3.8). The linkage of transfer oligosaccharide by amylomaltase from *E. coli* IFO 3816 was reported to be an α-type (Kitahara *et al*., 1989). Preliminary results showed that only trace amounts of products were observed when used soluble starch as glucosyl donor. We then tested for raw tapioca, pea and corn starches as glucosyl donors, and higher amounts of products were obtained. This suggested that the amylomaltase from recombinant *Corynebacterium glutamicum* ATCC 13032 favored high DP of glucosyl donor molecule for transglycosylation reaction. The products were less in amount but there were few more types with some larger than the products of CGTase (Figure 3.3 and Figure 3.8). Accordingly, the transfer products of all reactions were analyzed by HPLC, the total transglycosylation yield from the raw tapioca starch was the highest among different glucosyl donors. This should be due to higher solubility of raw tapioca starch when compared with pea and corn starch. The use of amylomaltase for the synthesis of maltosylsucrose has never been reported. However, the thermostable amylomaltase from *Thermotoga maritima* was used to combine with a maltogenic amylase from *Bacillus stearothermophilus* in the production of isomalto-oligosaccharides from starch resulted in a reduction of reaction time, and a higher yield of isomalto-oligosaccharides (Lee *et al*., 2002).

4.6 Optimization of transglycosylation reaction of amylomaltase

In order to produce higher yield of maltosylsucrose from amylomaltase catalysis, transglycosylation reaction was optimized. The total yield of transfer product was increased about seven fold from 11.7% (Table 3.9) to 81.7% (Table 3.12) after optimization.

In the first step of optimization, amylomaltase was incubated with sucrose concentrations from 0-2.5% and 2.5% glucosyl donor (raw tapioca starch) and then the transfer products were analyzed by HPLC. We found that at 0.25% sucrose, only two products were observed at Rt 19 and 20 min (Table 3.9). When increased sucrose concentration to 1.0-1.5 %, more types of products were observed at Rt 17, 18, 22, 24, 26 and 30 min. At high sucrose concentration of 2.0-2.5%, the additional product at Rt 34 min was obtained. The results suggested that when sucrose concentration was excess, small products similar to the products from CGTase were obtained. After that, the glucosyl donor concentrations were optimized. The results indicated that, at higher concentrations of glucosyl donor (0.5-2.5%) larger products at 17-19 min were obtained in addition to smaller products at Rt 20-34 min (Table 3.10). Also when using enzyme higher than 0.25 U/ml, the additional products at Rt 16 min were observed. However, when increased enzyme concentrations over 5 U/ml, the products with high DP (Rt 16-17 min) were decreased which might be resulted from the influence of the hydrolysis activity of amylomaltase. At longer incubation time (18-72 hours) the main products were smaller (Rt 26-34 min) comparing to at shorter incubation time (6 and 12 hours) at which higher DP products (Rt 22-26 min and 24-30 min, respectively) were observed as main products (Table 3.12).These results suggested that the ratio of larger to smaller tranfer products could be controlled by adjusting the concentrations of sucrose, glucosyl donor, and enzyme together with the incubation time. It has been reported that amylomaltase prefers to transfer glucose units in multiples, and has low affinity for the transfer of single glucosyl units (Palmer and Ryman, 1968).

Our result suggested for the first time that amylomaltase was able to synthesize maltosylsucrose with the ratio of components different from those found in CGTase reaction (Table 4.1). The obtained yield after optimization was in the order of Rt 34 ≈ Rt 30 > Rt 26 > Rt 24 > Rt 22 > Rt 20 > Rt 19 ≈ Rt 18 > Rt 17 ≈ Rt 16 (later by MS, proved to be $G_2F \approx G_3F > G_4F > G_5F > G_6F > G_7F > G_8F \approx G_9F > G_{10}F \approx G_{11}F$). While the yield for CGTase was in the order of $G_5F \approx G_4F \approx G_3F > G_2F > G_7F \approx G_8F > G_9F$. Higher proportion of large products ($\geq G_7F$) could be obtained at lower amylomaltase concentration (5 U/ml in the before optimization condition). Also for CGTase, at especially low sucrose, higher proportion of G_6F and G_7F was obtained in the before as compared to after optimization condition. In contrast to commercial product produced from *Thermoanaerobacter* sp. CGTase, the ratio of larger to smaller components were higher in the products from amylomaltase. There are a few adventages of having high proportion of large oligosaccharides.

Larger oligosaccharides have more pronounced prebiotic effects than shorter ones e.g. inulin (long chain fructo-oligosaccharide) was slower fermented and higher prebiotic potency makes inulin a more interesting compound than short chain fructo-oligosaccharide to beneficially influence the microbial community from colon regions, the prebiotic effectiveness of inulin-type fructans not only depends on the dietary dosage, but also on the DP (Van De Wiele, *et al*. 2007; Van Loo, 2004). In addition, when DP of oligosaccharides were increased, the sweetness was decreased. Low sweet sugars are nutraceuticals, suitable for health food.

Table 4.1 The comparison of products and yields from CGTase and amylomaltase reactions before and after optimization

Oligosaccharides		CGTase (% product yield)		Amylomaltase (% product yield)	
Rt (min)	Type	Before optimization[a]	After optimization[b]	Before optimization[c]	After optimization[d]
34	G_2F	9.59	14.1	5.71	14.0
30	G_3F	1.30	16.3	7.30	13.9
26	G_4F	22.1	18.2	6.87	11.9
24	G_5F	4.17	17.3	6.84	9.81
22	G_6F	-[e]	-[e]	6.68	7.81
20	G_7F	31.6	11.9	5.65	6.20
19	G_8F	30.7	11.7	4.70	5.46
18	G_9F*	-	9.11	3.87	5.45
17	$G_{10}F$*	-	-[f]	3.14	3.86
16	$G_{11}F$*	-	-[f]	2.77	3.20

[a]0.05% (w/v) sucrose and 5% (w/v) soluble tapioca starch incubated with 200 U/ml CGTase for 24 h.

[b]2.5% (w/v) sucrose and 20% (w/v) soluble tapioca starch incubated with 400 U/ml CGTase for 18 h.

[c]2.0% (w/v) sucrose and 2.5% (w/v) raw tapioca starch incubated with 5 U/ml amylomaltase for 24 h.

[d]2.0% (w/v) sucrose and 2.5% (w/v) raw tapioca starch incubated with 9 U/ml amylomaltase for 48 h.

[e]The product of CGTase at 22 min was also appeared in the control reaction.

[f]The products $G_{10}F$ and $G_{11}F$ of CGTase might be obtained, at the Rt 16, 17 min but they merged with the peak of glucosyl donor.

*The types are estimated from Rt compared with $\leq G_8F$ which are confirmed by MS data.

4.7 Larger scale preparation and isolation of maltosylsucrose products

In order to characterize maltosylsucrose products, preparation of higher amount of transfer products was performed by increasing reaction volume from 0.5 ml to 25 ml. The products were then isolated by Biogel-P2, a size exclusion gel column. Biogel-P2 gels are porous polyacrylamide beads prepared by copolymerization of acrylamide and N,N'-methylene-bis-acrylamide. The gels are extremely hydrophilic and essentially free of charge, and provide efficient, gentle gel filtration of sensitive compounds. High resolution is assured by consistent narrow distribution of bead diameters (Bio-Rad Laboratories, 2000). From Biogel-P2 column profile of maltosylsucrose products obtained from CGTase and amylomaltase eluted with water (Figure 3.15, 3.18), the high molecular weight molecules were eluted first. The fractions containing sugars were followed by the phenol-sulfuric method (Dubois *et al*., 1956).

For the product profile of CGTase, in the fractions range of 60-90 were glucosyl donors (soluble tapioca starch), as confirmed by retention time in HPLC. The fractions from 90-142 displayed nine peaks of sugars reacted with phenol-sulfuric acid (Figure 3.15). And the results of HPLC indicated that each peak was made up of a mixture of different products and DP could be estimated from the retention time compared with those of sucrose and glucose/fructose. The larger products were earlier eluted and sucrose peak was lastly eluted as two peaks of glucose (Rt 41 min) and fructose (Rt 44 min) due to hydrolysis at high column temperature (80°C). From HPLC analysis, the main products of CGTase after optimization were $G_5F \approx G_4F \approx G_3F > G_2F$ with the ratio of 1.3:1.2:1.2:1.0 (Table 4.1).

The profile of the transfer products of amylomaltase, in the fractions range of 60-90 were glucosyl donor (raw tapioca starch) at Rt 13min and Rt 15min. Oligosaccharide products were observed in the fraction range of 90-142. When each peak was analyzed by HPLC, the main products at Rt 19 and 20 min, 20 and 22 min, 22 and 24 min, 24 and 26 min were found in peaks C, D, E and F, respectively, and

the main products at Rt 26, 30 and 34 were found in peak G, H and I, respectively (Figure 3.18). Each maltosylsucrose product was separated due to different in size, the larger products (lower Rt) were earlier eluted and the last eluted peak was sucrose splitted into glucose and fructose. From HPLC analysis, the main products of amylomaltase after optimization were $G_2F \approx G_3F > G_4F > G_5F$ with the ratio of 1.0:0.9:0.8:0.7 (Table 4.1).

In previous report, maltosylsucrose products were purified by a preparative Biogel-P2 column. Detection of products was performed by HPEAC analysis of each fraction. Each pure fraction was then collected and lyophilized (Monthieu *et al*., 2003). The sucrose transfer products were isolated by alkali-degradation followed by charcoal column chromatography to remove the reducing sugars, then purified by ion exchange and Biogel P-2 gel permeation chromatography (Lee *et al*., 2003).

4.8 Characterization of products

The molecular weight of major synthesized products by amylomaltase was elucidated by ESI-ToF mass spectrometry. The molecular weights of the products at Rt 34, 30, 26, 24, 22 and 20 were estimated to be 504, 666, 828, 990, 1152 and 1314 dalton, respectively (Figure 3.19-3.21). These sizes were corresponded to a trisaccharide (maltosyl fructoside, G_2F), a tetrasaccharide (maltotriosyl fructoside, G_3F), a pentasacharide (maltotetraosyl fructoside, G_4F), a hexasaccharide (maltopentaosyl fructoside, G_5F), a heptasaccharide (maltohexaosyl fructoside, G_6F), and an octasaccharide (maltoheptaosyl fructoside, G_7F).

By comparison of retention time in HPLC of the products from CGTase and amylomaltase, the main products of CGTase at Rt 24, 26, 30 and 34 min corresponded to G_5F, G_4F, G_3F and G_2F respectively. The main products of maltosylsucrose from *Paenibacillus* sp. RB01 CGTase in this study were different from those reported in *Thermoanaerobacter* sp CGTase reaction in which G_2F and G_3F were the major types (Martín *et al*., 2004; Monthieu *et al*., 2003). While the main

products of amylomaltase were Rt 24, 26, 30 and 34 min, which were corresponded to G_5F, G_4F, G_3F and G_2F, respectively. From previous report, the high DP maltosylsucrose became glucosyl donors because of excess of sucrose and led mainly to the synthesis of G_2F and G_3F. These observations explain why the main transglycosylation products they obtained are G_2F and G_3F and imply that the production of more homogeneous oligosaccharides mixture depends on several factors (Soro *et al*., 2007).

4.9 Determination of biological properties of maltosylsucrose

S. mutans is known to be involved in dental caries in humans and experimental animals by synthesizing extracellular and water-insoluble glucans from sucrose by GTase and by releasing acids from various fermentable sugars (Lee *et al*., 2003). The cariogenicity of sucrose has been explained by the fact that the sugar is a good substrate for acid fermentation by oral microorganisms and is also a good substrate for the synthesis of insoluble glucan, causing accumulation of dental plaque on a tooth surface (Imai *et al*., 1984).

The tests of the inhibitory effects of our maltosylsucrose products on the water insoluble glucan synthesis, the fermentation of sugar and plaque formation were performed and compared with sucrose and commercial coupling sugar. The result showed that the inhibitory effects on GTase of the mixture of maltosylsucrose products and commercial coupling sugar on the synthesis of water insoluble glucan were reduced by about 16-19% as compared to that formed by sucrose alone (Table 3.15). The value was lower than 50% reduction in the previous report. This inhibition in the synthesis of insoluble glucan was suggested to be concerned with an acceptor reaction because maltosylsucrose product is consisted of sucrose and several DP of maltosylsucrose (Lee *et al*., 2003). Fifty percent of reduction in plaque formation was also observed. When the inhibitory effect of maltosylsucrose on the acid fermentation was investigated, the final pH of the medium with the maltosylsucrose mixture was pH 5.70 and that of commercial coupling sugar was pH 5.62 which were higher than that of sucrose (pH 4.37). This is in agreement with those observed with various

sugars (Imai *et al*., 1984). In terms of damaging effect on tooth surface, pH has to below 5.5 (Decker and van Loveren, 2003). Furthermore, our maltosylsucrose and commercial coupling sugar were lower in the ability to induce aggregation of cells, comparing with sucrose. It is likely that sucrose-induced aggregation would also be affected by more accessible dextran receptors which could bind newly synthesized glucan and enhance cell-to-cell binding (Otake *et al*., 1981).

Maltosylsucrose was reported to be more effective as a substitute for sucrose than as an inhibitor of sucrose utilization in limiting *S. mutans*-induced caries. *S. mutans* 6715 metabolized maltosylsucrose for growth and acid production, but utilization for polysaccharide production, in vitro plaque formation, cellular aggregation, and adherence of cells to glass surfaces were poor (Ikeda *et al*., 1978).

The inhibitory effect of different ratio of sucrose to maltosylsucrose on GTase activity was also performed. The addition of the maltosylsucrose mixture to sucrose inhibited the water insoluble glucan synthesis by GTase of *S. mutans.* When the ratio was 1:4, the inhibition was about 50%. The results were similar when mixed sucrose with commercial coupling sugar (Table 3.16). Similar result was reported in the case of trehalose which could inhibit the water insoluble glucan synthesis by GTase of *S. mutans* by 10-60% when similar ratio of sucrose to trehalose was used (Neta *et al*., 2000). Imai *et al*. (1984) reported that maltose and maltosylfructoside could also inhibit GTase synthesis, and might act as acceptors in the transglycosylation reaction.

In conclusion, cyclodextrin glycosyltransferase from *Paenibacillus* sp. RB01 and recombinant amylomaltase from *Corynebacterium glutamicum* were able to synthesize maltosylsucrose from sucrose and a glucosyl donor. The composition and yield of maltosylsucrose mixture could be controlled by varying amounts of sucrose, glucosyl donor, enzyme and incubation time. From the result of inhibitory effect on GTase, maltosylsucrose products were not only low cariogenic but also anticariogenic oligosaccharides.

CHAPTER V

CONCLUSIONS

1. CGTase from *Paenibacillus* sp. RB01 was partially purified by starch adsorption with a 22.9 fold increase in specific activity and a 56.1 percent yield.

2. The recombinant amylomaltase from *Corynebacterium glutamicum* ATCC 13032 was 15.4 fold purified by HisTrap affinity column with 47.3 percent yield.

3. CGTase was able to synthesize maltosylsucrose using sucrose acceptor and various glucosyl donors e.g. soluble starch from potato and tapioca starch, β-CD and maltoheptaose. These substrates were equally effective as glucosyl donor.

4. Raw tapioca, pea and corn starch were used as glucosyl donor for the synthesis of maltosylsucrose by amylomaltase. Raw tapioca starch was the best glucosyl donor.

5. The optimal condition for the synthesis of maltosylsucrose from CGTase was determined as 2.5% (w/v) sucrose, 20% (w/v) tapioca starch (soluble), 400 U/mL (dextrinizing unit) of CGTase at 40°C for 18 hours and that for amylomaltase was 2.0% (w/v) sucrose, 2.5% (w/v) tapioca starch (raw), 9 U/ml (disproportionation unit) of amylomaltase at 30°C for 48 hours.

6. After optimization, the main products of CGTase were G_5F, G_4F, G_3F and G_2F with the ratio of 1.3:1.2:1.2:1.0. The overall product yield was about 98%. While, the main products of amylomaltase were G_2F, G_3F, G_4F and G_5F with the ratio of 1.0:0.9:0.8:0.7 and the overall product yield of 82%.

7. Larger maltosylsucrose products (G_7F, G_8F, > G_8F) could be obtained in higher yield and or higher ratio at low concentrations of sucrose and enzyme.

8. The tests of the inhibitory effects of the maltosylsucrose on the water insoluble glucan synthesis, the fermentation of sugar and plaque formation, indicated that the maltosylsucrose products were able to inhibit the activity of GTase from *Streptococcus mutans*.

9. Maltosylsucrose products also inhibited the synthesis of water insoluble glucan by sucrose.

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APPENDICES

Appendix A Preparation for polyacrylamide gel electrophoresis

1) **Stock reagents**

1.5 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane	18.2 g

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

2.0 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

0.5 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane	6.06 g

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

1.0 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

Solution B (SDS-PAGE)

2 M Tris-HCl pH 8.8	75 ml
10% SDS	4 ml
Distilled water	12 ml

Solution C (SDS-PAGE)

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

2) Denaturing PAGE (SDS-PAGE)

7.5% separating gel

40% Acrylamide solution	1.40 ml
Solution B (SDS-PAGE)	1.87 ml
Distilled water	4.12 ml
10% $(NH_4)_2S_2O_8$	100 µl
TEMED	10 µl

5.0% separating gel

40% Acrylamide solution	0.625 ml
Solution B (SDS-PAGE)	1.25 ml
Distilled water	3.07 ml
10% $(NH_4)_2S_2O_8$	50 µl
TEMED	5 µl

Sample buffer

1 M Tris-HCl pH 6.8	0.6 ml
50% Glycerol	5.0 ml
10% SDS	2.0 ml
2-Mercaptoethanol	0.5 ml
1% Bromophenol blue	1.0 ml
Distilled water	0.9 ml

One part of sample buffers was added to four parts of sample. The mixture was heated for 5 minutes in boiling water prior to loading to the gel.

Electrophoresis buffer, 1 litre

Tris (hydroxymethyl)-aminomethane	3.0 g
Glycine	14.4 g
SDS	1.0 g

Adjusted volume to 1 litre with distilled water (pH should be approximately 8.3)

Appendix B Preparation for buffer solution

0.1 M Sodium acetate pH 5.5

CH3COONa	0.61 g

Adjusted volume to 100 ml with distilled water. Adjusted to pH 5.5

0.2 M Phosphate pH 6.0

KH_2PO_4	3.28 g
K_2HPO_4	0.16 g
Distilled water	100 ml

50 mM Phosphate pH 6.0

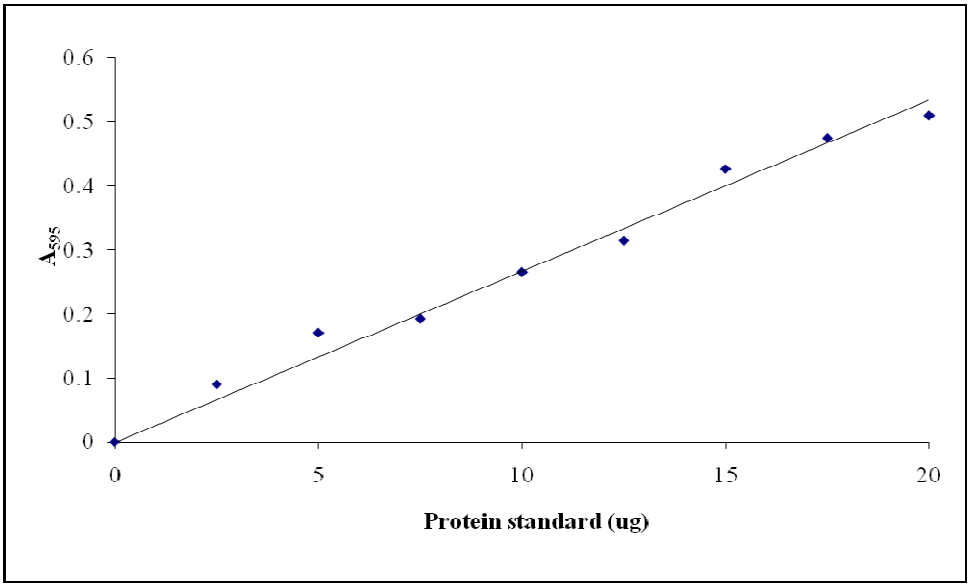
KH_2PO_4	0.82 g
K_2HPO_4	0.04 g
Distilled water	100 ml

0.2 M Tris-Glycine NaOH pH 8.0

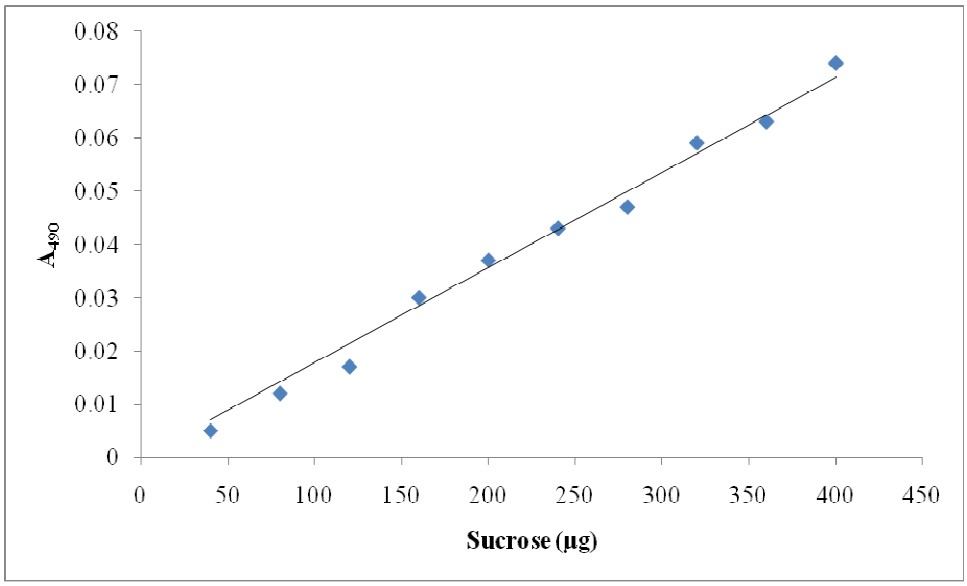
Glycine	1.5 g

Adjusted to pH 8.0 by 1 M NaOH and adjusted volume to 100 ml with distilled water.

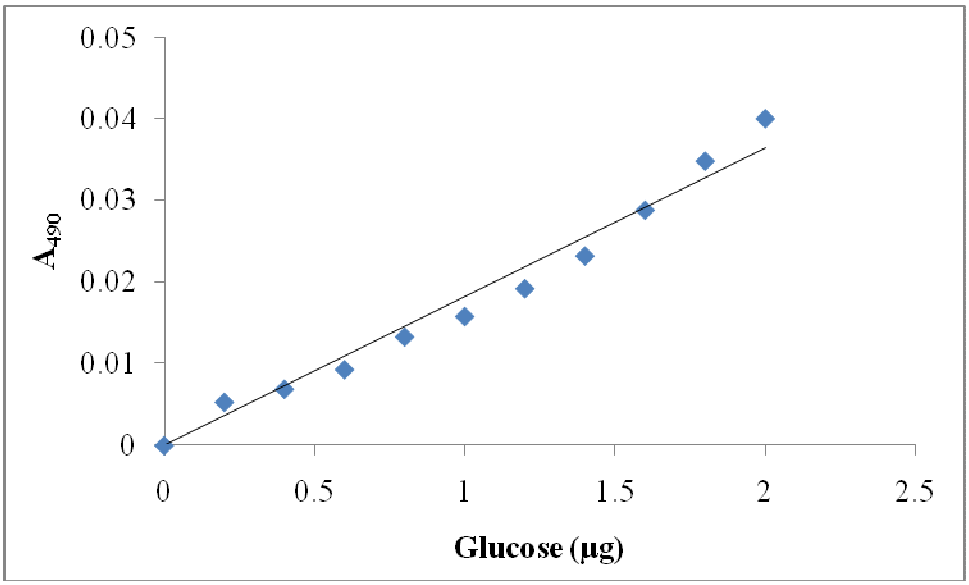
Appendix C Standard curve for protein determination by Bradford's method



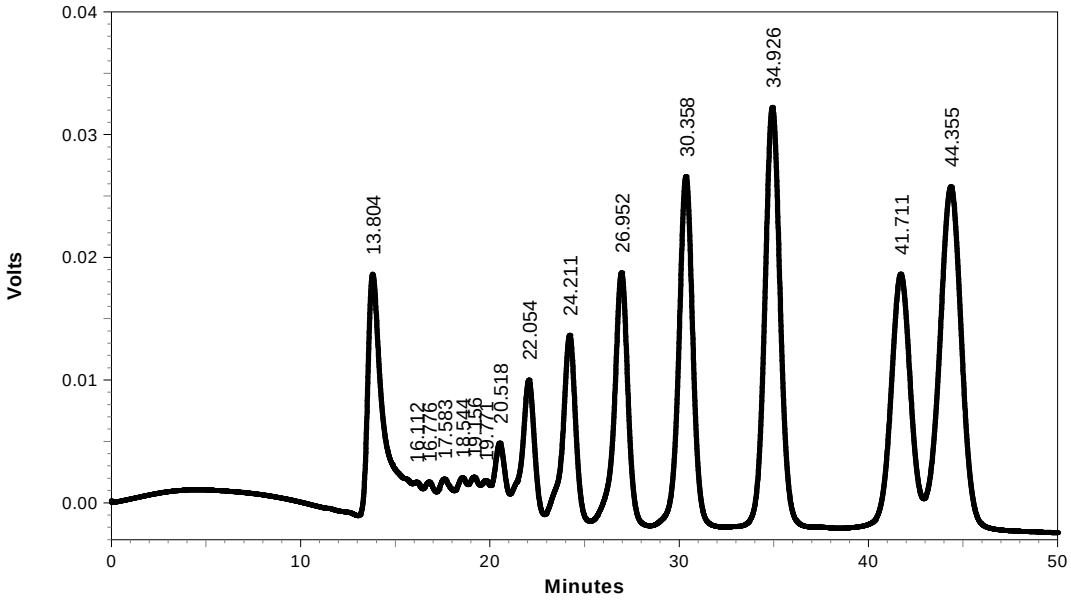
Appendix D Standard curve of sucrose concentration by phenol-sulfuric method



Appendix E Standard curve of glucose concentration by phenol-sulfuric method



Appendix F HPLC chromatogram of commercial coupling sugar



BIOGRAPHY

Miss Siriwipa Saehu was born on July 16th, 1983. She graduated with the Bachelor's degree of Science from the Department of Chemistry at Silpakorn University in 2005, and continued studying for the Master degree of Science in Biochemistry program, Faculty of Science at Chulalongkorn University in 2008.