การสังเคราะห์เอพิแคทีชินไกลโคไซด์โดยปฏิกิริยาโยกย้ายหมู่ไกลโคซิลที่จำเพาะของไซโคลเดกซ์ทรินไกลโคซิลทรานสเฟอเรส

นางสาวพรพรรณ อร่ามแสงเทียนชัย

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

SYNTHESIS OF EPICATECHIN GLYCOSIDES VIA SPECIFIC TRANSGLYCOSYLATION OF CYCLODEXTRIN GLYCOSYLTRANSFERASE

Miss Pornpun Aramsangtienchai

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biochemistry
Department of Biochemistry
Faculty of Science
Chulalongkorn University
Academic Year 2007
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Thesis Title	SYNTHESIS OF EPICATECHIN GLYCOSIDES VIA SPECIFIC TRANSGLYCOSYLATION OF CYCLODEXTRIN GLYCOSYLTRANSFERASE
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Field of Study	Biochemistry
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พรพรรณ อร่ามแสงเทียนชัย : การสังเคราะห์เอพิแคทีชินไกลโคไซด์โดยปฏิกิริยาโยกย้ายหมู่ไกลโคซิลที่จำเพาะของไซโคลเดกซ์ทรินไกลโคซิลทรานสเฟอเรส. (SYNTHESIS OF EPICATECHIN GLYCOSIDES VIA SPECIFIC TRANSGLYCOSYLATION OF CYCLODEXTRIN GLYCOSYLTRANSFERASE) อ. ที่ปรึกษา: รศ. ดร. เปี่ยมสุข พงษ์สวัสดิ์, อ. ที่ปรึกษาร่วม: ผศ. ดร. วรินทร ชวศิริ, 113 หน้า.

ในการทดลองนี้ได้ศึกษาการเร่งปฏิกิริยาการโยกย้ายหมู่ไกลโคซิลของเอนไซม์ไซโคลเดกซ์ทรินไกลโคซิลทรานสเฟอเรส (CGTase) ที่บริสุทธิ์บางส่วนจากแบคทีเรีย *Paenibacillus* sp.RB01 ไปยังตัวรับซึ่งเป็นแคทีชินชนิดต่างๆ ผลจากการวิเคราะห์ด้วยโครมาโทกราฟีแบบแผ่นบางพบว่า เมื่อใช้ตัวรับเป็น (+) แคทีชิน (-) เอพิแคทีชิน และ (-) เอพิแกลโลแคทีชิน แกลเลต โดยมีตัวให้หมู่กลูโคซิลเป็นบีตา-ไซโคลเดกซ์ทริน จะพบผลิตภัณฑ์กลูโคไซด์เกิดขึ้น 3 ถึง 4 ชนิด โดยเอนไซม์มีความจำเพาะต่อตัวรับหมู่กลูโคซิล (-) เอพิแกลโลแคทีชินแกลเลตมากที่สุด ขณะที่ความจำเพาะต่อตัวให้ทั้ง 3 ชนิด ได้แก่ แป้ง บีตา-ไซโคลเดกซ์ทริน และมอลโทเฮปตะโอส มีความใกล้เคียงกัน จากการวิเคราะห์ผลิตภัณฑ์ที่เกิดขึ้นด้วยเทคนิค HPLC พบว่าภาวะที่เหมาะสมต่อการสังเคราะห์ผลิตภัณฑ์กลูโคไซด์โดยมีเอพิแคทีชินเป็นตัวรับ คือ การบ่มเอนไซม์ 80 ยูนิตต่อมล. กับ 1.0% (w/v) บีตา-ไซโคลเดกซ์ทริน และ 0.5% (w/v) เอพิแคทีชิน ใน 10 มิลลิโมลาร์ อะซีเตตบัฟเฟอร์ pH 6.0 ที่ 50 ° ซ เป็นเวลา 24 ชม. ผลิตภัณฑ์รวมที่ได้คิดเป็น 18.07% ของสารตั้งต้นที่ใช้ทั้งหมด โดยมีผลิตภัณฑ์แต่ละชนิดที่ค่า Rt 27, 30, 45 และ 48 นาที คิดเป็น 1.44%, 3.39%, 4.18% และ 8.33% ตามลำดับ ในระหว่างการหาภาวะที่เหมาะสมต่อการสังเคราะห์กลูโคไซด์พบว่า ในภาวะด่าง อุณหภูมิสูง ตลอดจนการบ่มที่ระยะเวลานาน สารตั้งต้นเอพิแคทีชินสามารถเปลี่ยนเป็นแคทีชินได้โดยปฏิกิริยาเอพิเมอไรเซชัน เมื่อเพิ่มปริมาณการสังเคราะห์กลูโคไซด์และแยกผลิตภัณฑ์ด้วยคอลัมน์ Sephadex LH-20 และ คอลัมน์ C18-reversed phase จะได้ผลิตภัณฑ์หลักที่เวลา 48 นาที คิดเป็น 5.6% เมื่อทำการพิสูจน์โครงสร้างของกลูโคไซด์หลัก 2 ชนิด ด้วยเทคนิค MS และ NMR พบว่าผลิตภัณฑ์หลักที่ Rt 48 นาที คือ epicatechin-3′-*O*-α-D-glucopyranoside และที่ Rt 45 นาที คือ epicatechin-3′-*O*-α-D-diglucopyranoside ซึ่งละลายน้ำได้เท่ากับ 95.0 และ 2.0 มก.ต่อมล. ขณะที่ epicatechin มีค่าการละลายเท่ากับ 4.9 มก.ต่อมล. ความสามารถในการต้านการเกิดสีน้ำตาลเมื่อฉายแสงยูวีของ epicatechin monoglucoside มีมากกว่า epicatechin diglucoside และ epicatechin เมื่อศึกษาสมบัติในการต้านอนุมูลอิสระของผลิตภัณฑ์ พบว่าค่าความเข้มข้นของ epicatechin, epicatechin monoglucoside และ diglucoside ที่ทำให้การดูดกลืนแสงของ DPPH ลดลงครึ่งหนึ่ง (IC_{50}) มีค่าเท่ากับ 25, 36 และ 46 ไมโครโมลาร์ ตามลำดับ

ภาควิชา ชีวเคมี ลายมือชื่อนิสิต .....

สาขาวิชา ชีวเคมี ลายมือชื่ออาจารย์ที่ปรึกษา .....

ปีการศึกษา 2550 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม .....

4872382423 : MAJOR BIOCHEMISTRY
KEY WORD: CLYCODEXTRIN GLYCOSYLTRANSFERASE / EPICATECHIN / BETA-CYCLODEXTRIN / EPICATECHIN GLUCOSIDES/ TRANSGLUCOSYLATION
PORNPUN ARAMSANGTIENCHAI : SYNTHESIS OF EPICATECHIN GLYCOSIDES VIA SPECIFIC TRANSGLYCOSYLATION OF CYCLODEXTRIN GLYCOSYLTRANSFERASE. THESIS ADVISOR: ASSOC. PROF. PIAMSOOK PONGSAWASDI, Ph.D., THESIS COADVISOR : ASST. PROF. WARINTHORN CHAVASIRI, Ph.D., 113 pp.

Transglycosylation of catechins by partially purified cyclodextrin glycosyltransferase (CGTase) from *Paenibacillus* sp.RB01 was investigated. Three to four glucoside products were obtained by TLC analysis when used (+) catechin, (-) epicatechin, or (-) epigallocatechin gallate as acceptor and beta-cyclodextrin as glucosyl donor. The enzyme showed higher acceptor specificity towards epigallocatechin gallate whereas the same specificity for donor substrate (starch, beta-cyclodextrin, maltoheptaose) was demonstrated. When the occurred products in the reaction were analyzed by HPLC, the optimum condition for transglucosylation with epicatechin as an acceptor was: incubation of 80 U/ml CGTase with 1.0% (w/v) beta-CD and 0.5% (w/v) epicatechin in 10 mM acetate buffer, pH 6.0 at 50 °C for 24 hours. The 18.07 % of overall product yield was obtained. The amounts of each product at Rt 27, 30, 45, and 48 minutes were 1.44%, 3.39%, 4.18%, and 8.33%, respectively. During optimization of transglucosylation reaction, epimerization of epicatechin to catechin was observed at alkaline pH, high temperature and longer incubation time. Reaction products were then prepared in larger scale and were isolated using Sephadex LH-20 and C18-reversed phase HPLC column. The 5.6% yield of major product at Rt48 min was obtained. The structures of the two major glucoside products elucidated by MS and NMR techniques were epicatechin-3'-*O*-α-D-glucopyranoside (for Rt48 min) and epicatechin-3'-*O*-α-D-diglucopyranoside (for Rt45 min). The water solubility of epicatechin, the monoglucoside and the diglucoside in water were 4.9, 95 and 2.0 mg/ml, respectively. While the browning resistance to UV irradiation of epicatechin monoglucoside was higher than epicatechin diglucoside and epicatechin. The antioxidant activity of the products was also determined by DPPH scavenging assay. It was found that the inhibitory concentrations at which the absorbance of DPPH was reduced by 50% (IC_{50}) of epicatechin, the monoglucoside and the diglucoside were 25, 36 and 46 μM, respectively.

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Field of study BIOCHEMISTRY Advisor's signature Piamsook Pongsawasdi

Academic year 2007 Co-advisor's signature Warinthorn Chavasiri

ACKNOWLEDGEMENTS

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

My gratitude is also expressed to my co-advisor, Assistant Professor Dr. Warinthorn Chavasiri, for his valuable suggestions, new ideas and insights.

Sincere thanks and appreciation are also extened to Professor Dr. Aran Incharoensakdi, Assistant Professor Dr. Kanoktip Packdibamrung and Dr. Manchumas Prousoontorn who serve as the members of the Master committees, for their helpful suggestions and comments.

My thanks also go to all staff members and friends of the Biochemistry Department, for their assistance and friendship.

Finally, the greatest gratitude is expressed to my parents for their encouragement, willpower and heartiness support throughout my life.

The financial support of the Asahi Glass Foundation of Japan and Ratchadaphiseksompote Endowment Fund to Starch and Cyclodextrin Research Unit are also gratefully acknowledged.

CONTENTS

PAGE

PAGE

PAGE

PAGE

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

LIST OF TABLES

TABLE **PAGE**

TABLE **PAGE**

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

LIST OF FIGURES

FIGURE **PAGE**

FIGURE **PAGE**

ABBREVIATIONS

A	absorbance
BSA	bovine serum albumin
C	catechin
CDs	cyclodextrin
CGTase	cyclodextrin glycosyltransferase
ºC	degree Celsius
Da	dalton
DPPH	2, 2-diphenyl-1-picryhydrazyl
EC	epicatechin
EGCg	epigallocatechin gallate
ESI-TOF-MS	Electrospray Ionization-Time of Flight Mass Spectrometry
g	gram
h	hour
HPLC	High Performance Liquid Chromatography
l	litre
µg	microgram
µl	microlitre
NMR	Nuclear Magnetic Resonance
M	molar
min	minute
ml	millilitre
MW	molecular weight
PAGE	polyacrylamide gel electrophoresis
Rf	relative mobility
Rt	retention time
SDS	sodium dodecyl sulfate
U	unit(s)
w/v	weight by volume

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER I

INTRODUCTION

1.1 Flavonoids

Flavonoids are a group of chemical compounds with low molecular weight phenylbenzopyrones. They are subclass of polyphenols, which are characterized as containing two or more aromatic rings, each bearing at least one aromatic hydroxyl and connected with a carbon bridge (Clifford, 2001). For flavonoids, this bridge consists of three carbons that combines with an oxygen and two carbons of one of the aromatic rings (A ring) to form a third 6-member ring (C ring) (Figure 1).

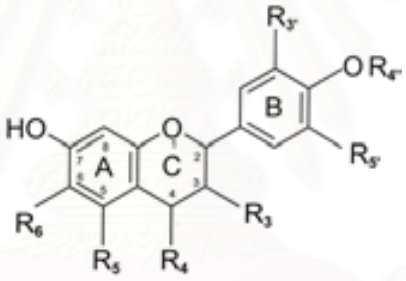


Figure 1 General structure and numbering pattern for common flavonoids
For most flavonoids, $R_{4'}$-H, R_5 -OH and R_6 -H. Exceptions include biochanin A, $R_{4'}$ -CH_3; formononetin, $R_{4'}$ -CH_3, R_5,R_6 -H; glycitein, R_5 -H, R_6 -OH; and hesperetin, $R_{4'}$ -CH_3. Additional individual flavonoids within each subclass are characterized by unique functional groups at R_3, $R_{3'}$, and $R_{5'}$ (Beecher, 2003)

Flavonoids are found in fruits, vegetables, nuts, seeds and beverages such as tea, coffee, beer and wine (Kandaswaani and Middleton, 1994). The flavonoids can be further divided into 6 subclasses: flavan-3-ol, flavanones, flavones, isoflavones, flavonols and anthocyanidins based on the connection of the B ring to the C ring and functional groups of the C ring (Table 1) (Beecher, 2003).

Table 1 Flavonoid subclasses, their chemical characteristics, names of prominent food flavonoids and typical food sources (Slightly modified from Beecher, 2003)

Flavonoid subclass	B-ring connection to C ring (position on C ring)	C-ring unsaturation	C-ring functional groups	Prominent food flavonoids[1]	Typical rich food sources
Flavan-3-ol	2	None	3-Hydroxy	(+)-Catechin (C) (+)-Gallocatechin (GC) (-)-Epicatechin (EC) (-)-Epigallo catechin (EGC)	Teas, red grapes and red wines
			3-O-gallate	(-)-Epicatechin-3-gallate (ECG) (-)-Epigallo catechin-3gallate (EGCG)	
Flavanones	2	None	4-Oxo	Eriodictyol Hesperetin Naringenin	Citrus foods
Flavones	2	2-3 Double bond	4-Oxo	Apigenin Luteolin	Green leafy spices, e.g., parsley
Isoflavones	3	2-3 Double bond	4-Oxo	Daidzein Genistein Glycitein Biochanin A Formononentin	Soybeans, soy foods and legumes
Flavonols	2	2-3 Double bond	3-Hydroxy, 4-Oxo	Isorhamnetin Kaempferol Myricetin Quercetin	Nearly ubiquitous in foods
Antho-cyanidins	2	1-2, 3-4 Double bonds	3-Hydroxy	Cyanidin Delphinidin Malvidin Pelargonidin Petunidin Peonidin	Red, purple and blue berries

[1] Flavonoids in highest concentration in foods and those reported in the USDA Database for the Flavonoid Content of Selected Foods. Total amount in food of each flavonoid reported as either aglycone or gallate form.

The flavonoids have potential beneficial effects on human health and they have been reported on antiviral (Nagai *et al*., 1992), antiplatelet (inhibition of aggregation and adhesion of platelets in blood) (Belinky *et al*., 1998), anti-inflammatory (Read, 1995), anti-allergic (Yamamura *et al*., 1998), antilipoperoxidant (Terao *et al*., 1994), and gastro-protective (Mojsil, 1999) properties. It is suggested that most of these biological effects are related to their antioxidant activity (Mojsilove and Kuchta, 2001). Flavonoids can exert their antioxidant activity by various mechanisms, e.g. by scavenging or quenching free radicals, by chelating metal ions, or by inhibiting enzymatic systems responsible for the generation of free radicals (Belinky *et al.*, 1998).

1.2 Catechins

Catechins are one group of flavonoids called flavan-3-ols. Chemically, catechins are polyhydroxylated flavonoids (Silvina and Cesar, 2000). Catechin was first isolated from the plant extract catechu, from which it derives its name. Heating catechin past its point of decomposition releases pyrocatechol, which explains the common origin of the names of these compounds. Although they are found in numerous plant species, the largest source of catechins in the human diet is from the tea-plant *Camellia sinensis* (Nagata and Sakai, 1984).

Catechins constitute about 25% of the dry weight of fresh tea leaf. Total catechins content vary widely depending on clonal variation, growing location, seasonal and light variation, and altitude. Also, the degree of fermentation greatly affects the quality and type of tea. According to the degree of fermentation, tea is classified into green tea (unfermented), oo-long tea (semi-fermented), and black tea (fully fermented) (Chu, 1997). Black tea leaves have a much lower content of catechins because they undergo extensive fermentation and the majority of the catechins are enzymatically oxidized to the major pigments of black tea leaves, theaflavin and thearubigin (Balentine *et al*., 1997). Catechins are also present in other plant species and in human diet such as chocolate, fruits, vegetables and red wine (Bravol, 1998) (Table 2).

Table 2 The content of catechins in some foods (modified from Manach *et al.*, 2004)

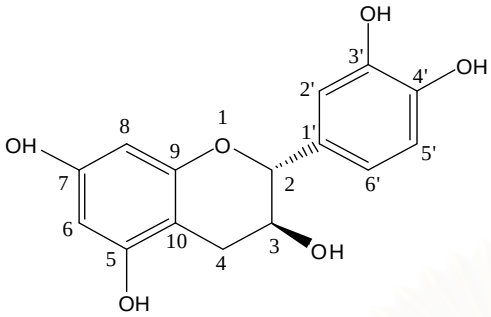
Foods	Catechins (mg per serving)	Catechins (mg per 100 g food)
Chocolate	23-30	46-61
Beans	70-110	35-55
Apricot	20-50	10-25
Cherry	10-44	5-22
Grape	6-35	3-17.5
Peach	10-28	5-14
Apple	20-86	10-43
Red raspberry	2-48	2-48
Strawberry	2-50	2-50
Blackberry	9-11	9-11
Green tea	20-160	10-80
Black tea	12-100	6-50
Red wine	8-30	8-30
Cider	8	4

1.2.1 Chemical and physicochemical properties of catechins

Catechins are a class of flavonols which are C15 compounds, and their derivatives are composed of two phenolic nuclei (A ring and B ring) connected by three carbon units (C-2, C-3, and C-4). This bent bridge, along with an oxygen atom, makes up the C ring. The flavanol structure of catechin (3, 3´, 4´, 5, 7-pentahydroxyflavan) contains two asymmetric carbon atoms at C-2 and C-3 (Ninomiya *et al*., 1997). The contents of green tea catechins are composed of six kinds of catechin and its derivatives: (+)-Catechin (C), (+)-Gallocatechin (GC), (-)-Epicatechin (EC), (-)-Epicatechin gallate (ECg), (-)-Epigallocatechin (EGC), (-)-Epigallocatechin gallate (EGCg). Epigallocatechin and gallocatechin contain an additional phenolic hydroxyl group when compared to epicatechin and catechin, respectively. Structures and physicochemical properties of catechins are shown in Figure 2. The most abundant tea catechins is in the order of (-)-EGCg > (-)-EGC > (-)-ECg > (-)-EC, respectively. While (+)-GC and (+)-C are usually trace or a minor component (Table 3).

Catechin is synthesized in tea leaves through malonic acid- and shikimic acid-metabolic pathways. Gallic acid is derived from an intermediary product produced in the shikimic acid-metabolic pathway (Figure 3) (Nakabayashi, 1991).

Catechins exist as two geometrical isomers, catechin in a *trans* form and epicatechin in a *cis* form, based on different configuration of 3', 4'- dihydroxyphenyl and hydroxyl groups at the 2- and 3-positions of the C-ring. The interesting uniformity of the occurrence of these natural products in the forms (+)-catechin, (-)-epicatechin, (+)-gallocatechin, and (-)-epigallocatechin is seen to residue in the identity of their configurations at the 2-position, and all these compounds may be defined as (2R)-flavan derivatives according to the method for specification of absolute configuration proposed by Cahn, Ingold, and Prelog R/S nomenclature. The sign of rotation, however, is (+) or (-) according to the configuration (3s or *3R* respectively) of the 3- hydroxyl group (Table 4). The configurational prefixes *D* and *L* decided by the position of the hydroxyl group in the Fischer projections have little significance when applied to catechin (Birch *et al*., 1957). (-)-Epicatechin

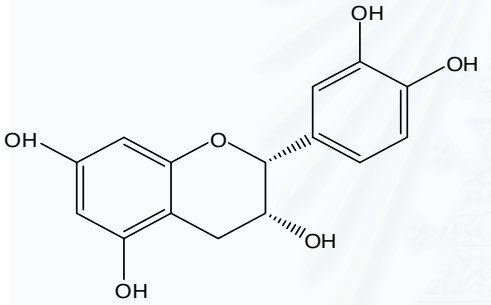


(+)-Catechin ((+)-C)

$C_{15}H_{14}O_6$, MW 290

m.p. 176 °C

[α] D + 18 °



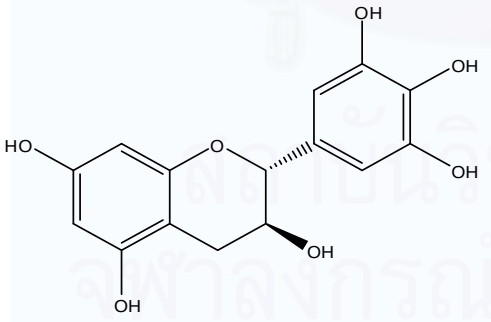
(-)-Epicatechin ((-)-EC)

$C_{15}H_{14}O_6$, MW 290

m.p. 242 °C

[α] D - 69 °

λ_{max} 280, ε_{max} 3,300



(+)-Gallocatechin ((+)-GC)

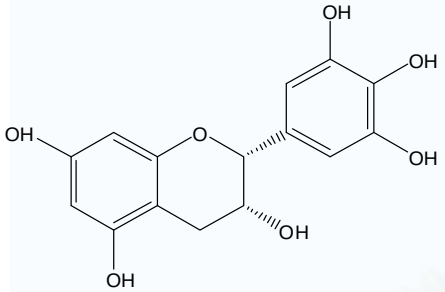
$C_{15}H_{14}O_7$, MW 306

m.p. 218 °C

[α] D + 15 °

λ_{max} 271, ε_{max} 1,290

Figure 2 Structure, molecular formula, molecular weight, melting point, optical rotation and maximum absorption wavelength of each component of green tea polyphenols (Hergert and Kurth, 1953; Bradfield and Penny, 1948; and Birch *et al*., 1957)

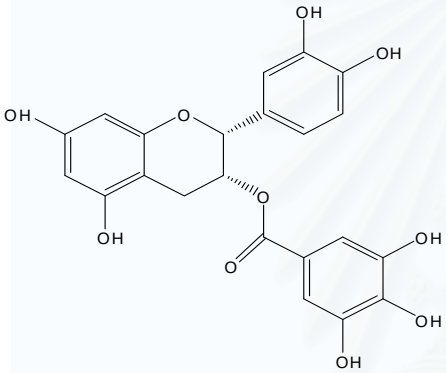


(-)-Epigallaocatechin ((-)-EGC)

$C_{15}H_{14}O_7$, MW 306

m.p. 218 °C

[α] D - 15 °



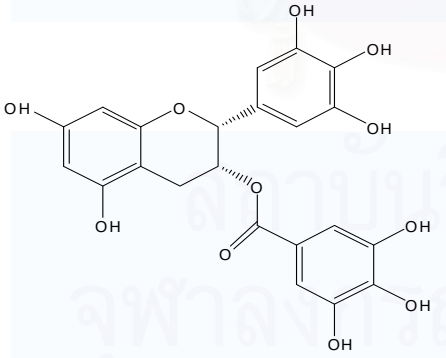
(-)-Epicatechin gallate ((-)-ECg)

$C_{22}H_{18}O_{10}$, MW 442

m.p. 253 °C

[α] D - 177°

λ_{max} 280, ε_{max} 13,600



(-)-Epigallocatechin gallate ((-)-EGCg)

$C_{22}H_{18}O_{11}$, MW 458

m.p. 254 °C

[α] D - 190°

Figure 2 Structure, molecular formula, molecular weight, melting point, optical rotation and maximum absorption wavelength of each component of green tea polyphenols (Hergert and Kurth, 1953; Bradfield and Penny, 1948; and Birch *et al*. 1957) (continued)

Table 3 Contents of several characteristic chemical components in leaves of genus *Camellia* and interspecific hybrids (g per 100 g dried leaves) (modified from Nagata and Sakai, 1984)

Species	Polyphenols*				
	C	EC	EGC	ECg	EGCg
I. *Thea*					
C. sinensis					
var. *sinensis*	0.07	1.13	2.38	1.35	8.59
var. *assamica*	0.02	1.44	0.35	3.35	12.10
C. taliensis	tr.**	0.58	0.8	1.9	6.84
C. irrawadiensis	0.03	0.72	0.12	0.67	0.21
II. *Camellia*					
C. japonica					
var. *japonica*	0.25	4.81	0	0	0
var. *decumbens*	2.04	3.57	0	0	0
C. reticulata	0.11	0.26	0	0	0
C. saluenensis	0.07	0.35	0	0	0
C. pitardii	0.25	6.64	0.46	0	0
III. *Paracamellia*					
C. sasanqua	0	0.02	0	0	0
C. oleifera	0.19	0	0	0	0
C. kissi (seedling)	tr.**	tr.**	0	0	0
IV. Hybrids					
C. sasanqua x C. sinensis	0.22	0.23	0.07	0.29	0.56
C. sinensis x C. Japonica	0.03	0.49	0.81	0.34	1.14
C. japonica x C. kissi	0	tr.**	0	0	0

* C-Catechin, EC-Epicatechin, EGC-Epigallocatechin, ECg-Epicatechin gallate, EGCg-Epigallocatechin gallate

** tr. – trace

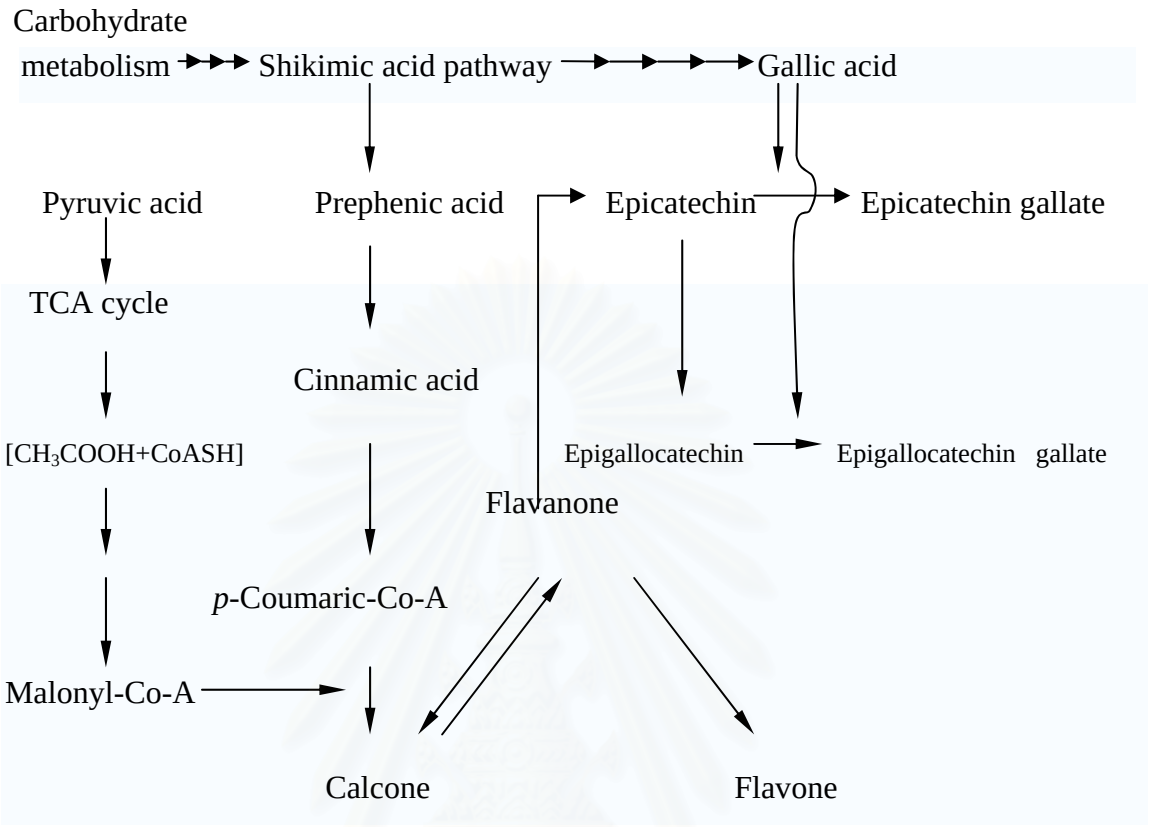


Figure 3 Biosynthesis pathways of catechins in tea (Nakabayashi, 1991)

Table 4 Hydroxylation patterns and absolute configurations of special green tea polyphenols (Birch *et al.*, 1957)

Compounds	Hydroxylation pattern	Absolute configuration
(+)-C	5, 7, 3', 4'	2R:3S
(-)-EC	5, 7, 3', 4'	2R:3R
(+)-GC	5, 7, 3', 4', 5'	2R:3S
(-)-EGC	5, 7, 3', 4', 5'	2R:3R

and (+)-catechin are the most common optical isomers found in nature. These isomers often exhibit different behavior in terms of pharmacological processes, therapeutic efficacy and biological processes (Tsuchiya, 2001).

Catechins have nucleophilic centers at C-6 and C-8 which are reactive with electrophilic specimens. They are highly chemically reactive, showing the properties of metal chelator, oxidative radicals scavenger, nitrosation inhibitor, etc. (Ninomiya *et al*., 1997). Catechins are flavonoids with bitter taste and have astringent property. The ester types of catechins, ECg and EGCg which are catechin gallates or gallic esters of the catechins, are stronger in bitterness and more astringent than EC and EGC (Chu and Juneja, 1997).

1.2.2 Biological activity of catechins

There are many papers published on the physiological and pharmacological functions of green tea. The antioxidant activity of catechins is widely used as a natural antioxidant for the prevention of oxidation of edible oils or minimization of discoloring of reddish foods (Koketsu, 1997). The green tea catechins have been shown to be more effective antioxidants than Vitamins C and E (Rice-Evans *et al*., 1995). However, the degree of antioxidant activity of individual catechins was examined by several antioxidative assay systems. The antioxidative activities of catechins were in the following order: (-)-ECg > (-)-EC, (-)-EGCg > (-)-EGC in the beta-carotene-linoleate model system (Amarowicz and Shahidi, 1995). On the other hand, the antioxidative activities of polyphenols on soybean oil or lard oil were in the order: (-)-EGCg > (-)-EGC > (-)-ECg > (-)-EC (Matsusaki and Hara, 1985). While the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging ability of the catechins was (-)-EGCg > (-)-ECg > (-)-EGC > (-)-EC (Koketsu, 1997). Although there is little difference in various assay systems examined, EGCg which is the major component among tea polyphenols and constitutes more than 50% of the total amount of tea catechins, shows the strongest antioxidative activity. EGCg is believed to be the most pharmacologically active tea catechins (Stagg and Millin, 1975).

Catechins were found to show several biochemical activities such as angiotensin converting enzyme inhibitory activity (Hara *et al*., 1987), regulation of plasma cholesterol level (Muramatsu *et al*., 1986), platelet aggregation inhibitory activity (Namiki *et al*., 1991), and inhibition of bacterial activity (Hara and Watanabe, 1989). They are effective against various strains of foodborne pathogenic bacteria, such as *Clostridium botulinum, Clostridium perfringens, Vibrio parahaemolyticus, Vibrio fluvialis, Vibrio metschnikovii, Staphylococcus aureus, Bacillus cereus, Plesiomonas shigelloides* and *Aeromonas sobria* while other organisms are less affected. Tea catechins inhibit expression of many viruses at concentrations less than 10 ppm, such as human papilloma virus, influenza virus in mice, pseudorabies virus, bovine herpes virus (Bidlack, 2001) and also inhibit HIV reverse transcriptase activity (Nakane and Ono, 1990).

In the laboratory, studies have shown that tea catechins act as powerful inhibitors of cancer growth in several ways. They scavenge oxidants before cell injuries occur, reduce the incidence and size of chemically induced tumors, and inhibit the growth of tumor cells. In studies of liver, skin and stomach cancer, chemically induced tumors were shown to decrease in size in mice that were fed green and black tea. They also can help to prevent tooth decay by killing the bacteria, *Streptococcus mutans,* causing dental plaque. Green tea contains fluoride and one cup of brewed green tea contains around 0.3-0.5 mg of fluoride which is an optimal level of fluoride according to dentists (Mayer, 2006).

Recently, catechin has been used as natural antioxidant in oils and fats against lipid oxidation, as supplements for animal feeds to improve animal health, as antimicrobial agents in foodstuffs, cosmetic products and as health functional ingredient in various foods and dietary supplements (El-Hady, 2007).

1.3 Cyclodextrins : Structures and characteristics

Cyclodextrins (CDs) are non-reducing, cyclic oligosaccharides composed of D-glucose units linked by α-1, 4 glycosidic bonds. Cyclodextrins mainly consist of 6, 7 or 8 glucose residues, commonly refer to α-cyclodextrin (Schardinger's α-dextrin,

cyclomaltohexaose, cyclohexaglucan, cyclohexaamylose, ACD, C6A), β-CD (Schardinger's β-dextrin, cyclomaltoheptaose, cycloheptaglucan, cycloheptaamylose, BCD, C7A), and γ-CD (Schardinger's γ-dextrin, cyclomaltooctaose, cyclooctaglucan, cyclooctaamylose, GCD, C8A), respectively (Figure 4) (Szejtli, 2004). The most important characteristic of CDs are summarized in Table 5 (Szejtli, 1988).

Because of the $^4C_1$ conformation of the glucopyranose units, all secondary hydroxyl groups are situated on one of the two edges of the ring, whereas all the primary ones are placed on the other edge. The ring is a conical cylinder, which is normally characterized as a doughnut or wreath-shaped truncated cone. The cavity is lined by the hydrogen atoms and the glycosidic oxygen bridges, respectively. The C-2-OH group of one glucopyranoside unit can form a hydrogen bond with the C-3-OH group of the adjacent glucopyranose unit. In the β-CD molecule, a complete secondary belt is formed by these H bonds; as a consequence, the β-CD is a rather rigid structure. This intramolecular H-bond formation is probably the explanation for the observation that β-CD has the lowest water solubility of all CDs. The H-bond belt is incomplete in the α-CD molecule, because one glucopyranose unit is in a distorted position. Therefore, instead of the six possible H bonds, only four can be established simultaneously, while the γ-CD is a non-coplanar and more flexible structure. Therefore, it is the most soluble of the all CDs. Hydrolysis rate of CDs increases in the order of α-CD< β-CD< γ-CD (Szejtli, 1998).

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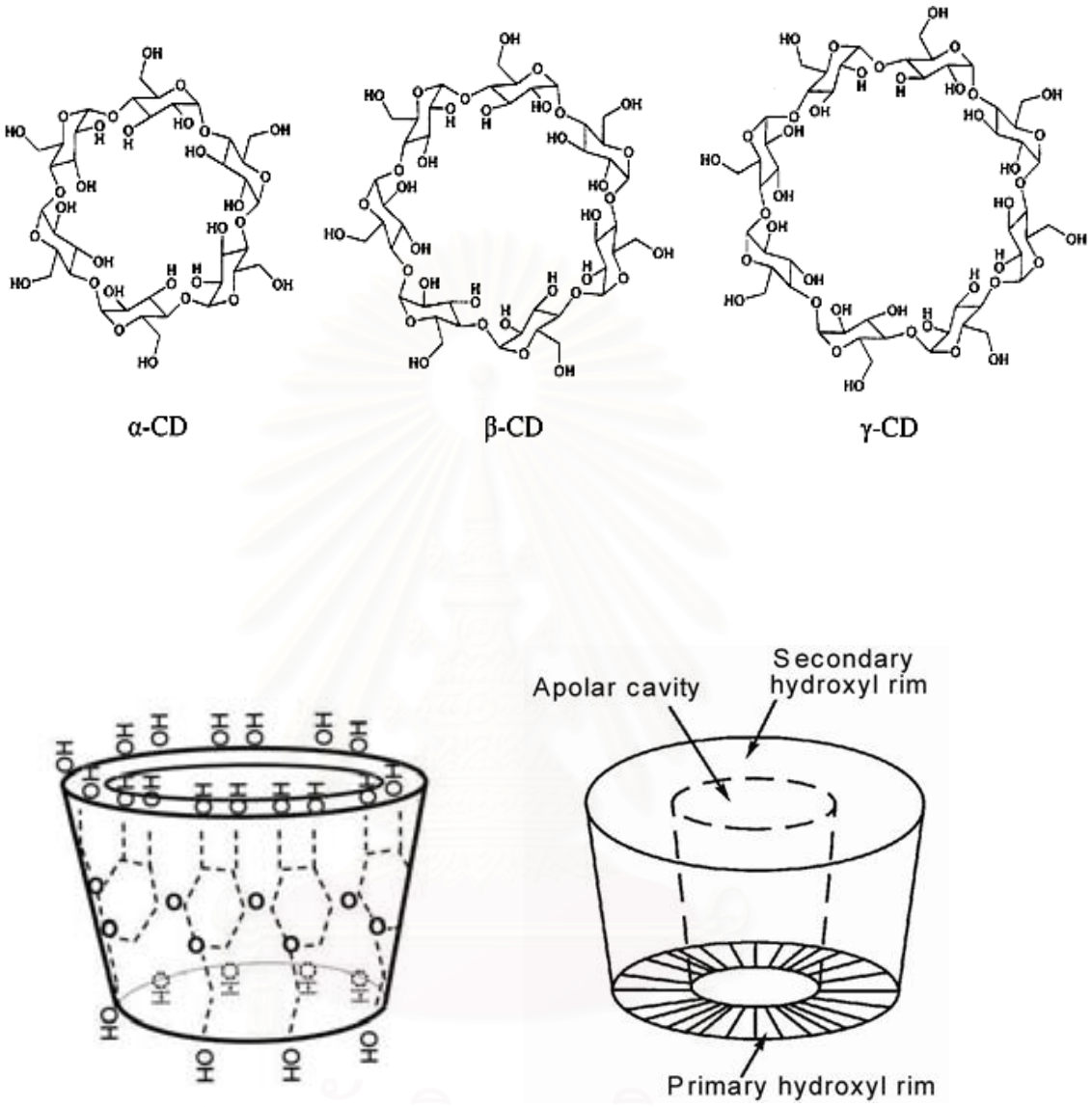

Figure 4 Chemical structure of cyclodextrins (CDs)

(a) α-, β- and γ-cyclodextrin consist of 6, 7 or 8 glucose units (Szejtli, 2004)

(b) Structure of cyclodextrin and glucose molecule arrangement (Cyclodextrin Technologies Development, Inc., 1990)

Table 5 Characteristics of α-, β-, γ-CDs (Szejtli, 1988)

Characteristics	α-CD	β-CD	γ-CD
Number of glucose unit	6	7	8
Molecular weight	972	1135	1297
Solubility in water (g/100 ml) at ambient temp	14.5	1.85	23.2
$[\alpha]_D$ 25 °C	150±0.5	162.5±0.5	177.4±0.5
Cavity dimensions			
Cavity diameter (A°)	4.7-5.3	6.0-6.5	7.5-8.3
Cavity depth (A°)	7.9±0.1	7.9±0.1	7.9±0.1
Cavity volume			
$(A°)^3$	174	262	427
ml per mol	104	157	256
ml per g	0.10	0.14	0.20
Crystal forms (from water)	hexagonal plates	monoclinic parallelograms	quadratic prisms
Hydrolysis by *A. oryzae* α-amylase	negligible	slow	rapid
pK (by potentiometry) at 25 °C	12.332	12.202	12.081

1.4 Cyclodextrin glycosyltransferase

Cyclodextrin glucosyltransferase (CGTase, 1, 4-α-D-glucan: 1, 4-α-D-glucopyranosyltransferase, EC 2.4.1.19) catalyzes the production of cyclodextrin from starch and related α-(1,4)-linked glucose polymers via a transglycosylation reaction. CGTase catalyzes four related reactions: cyclization, coupling, disproportionation and hydrolysis (Figure 5). The cyclodextins are produced via an intramolecular transglycosylation reaction in which CGTase cleaves an α-(1,4) bond in the starch molecules and concomitantly linking the reducing and non-reducing ends (cyclization). In the presence of a suitable acceptor, CGTase catalyzes intermolecular transglycosylation by transferring a glucose unit from the oligosaccharide donor to the acceptor (disproportionation reaction). The enzyme also catalyzes an opening of the rings of CDs and transfer of the linear maltooligosaccharides formed to oligosaccharide acceptors (coupling reaction) (Kitahata *et al*., 1974) When reducing end is not transferred to a carbohydrate acceptor but rather to a water molecule, the result is the hydrolysis of amylose or the linearization of cyclodextrin (hydrolysis reaction). CGTase is a member of the α-amylase family of glycosyl hydrolases. The enzymes in this group show a wide diversity in reaction specificities and many of them are active on starch. Whereas amylases generally hydrolyzed glycosidic bonds in the starch molecules, CGTase mainly catalyzes transglycosylation reaction, with hydrolysis being a minor activity (Van Der Veen *et al.,* 2000).

The CGTases are known to be produced by various genera of bacteria such as *Bacillus, Klebsiella, Pseudomonas, Brevibacterium, Thermoanaerobacterium, Corynebacterium, Micrococcus,* and *Clostridium* (Table 6). Most of the bacterial CGTases produce α-, β- and γ- CDs from starch in different ratios. There are few CGTases that produce predominantly α-CD or γ-CD (Bender, 1977; Kato and Horikoshi 1986, cited in Gawande *et al*., 1999). All the organisms producing CGTase that can convert starch predominantly into β-CD, also produce α-, γ-CD and malto-oligosaccharides in varying ratios. However, enzymes that can produce β-CD in higher ratios and small amounts of other CDs and malto-oligosaccharides are important (Gawande *et al*., 1999).

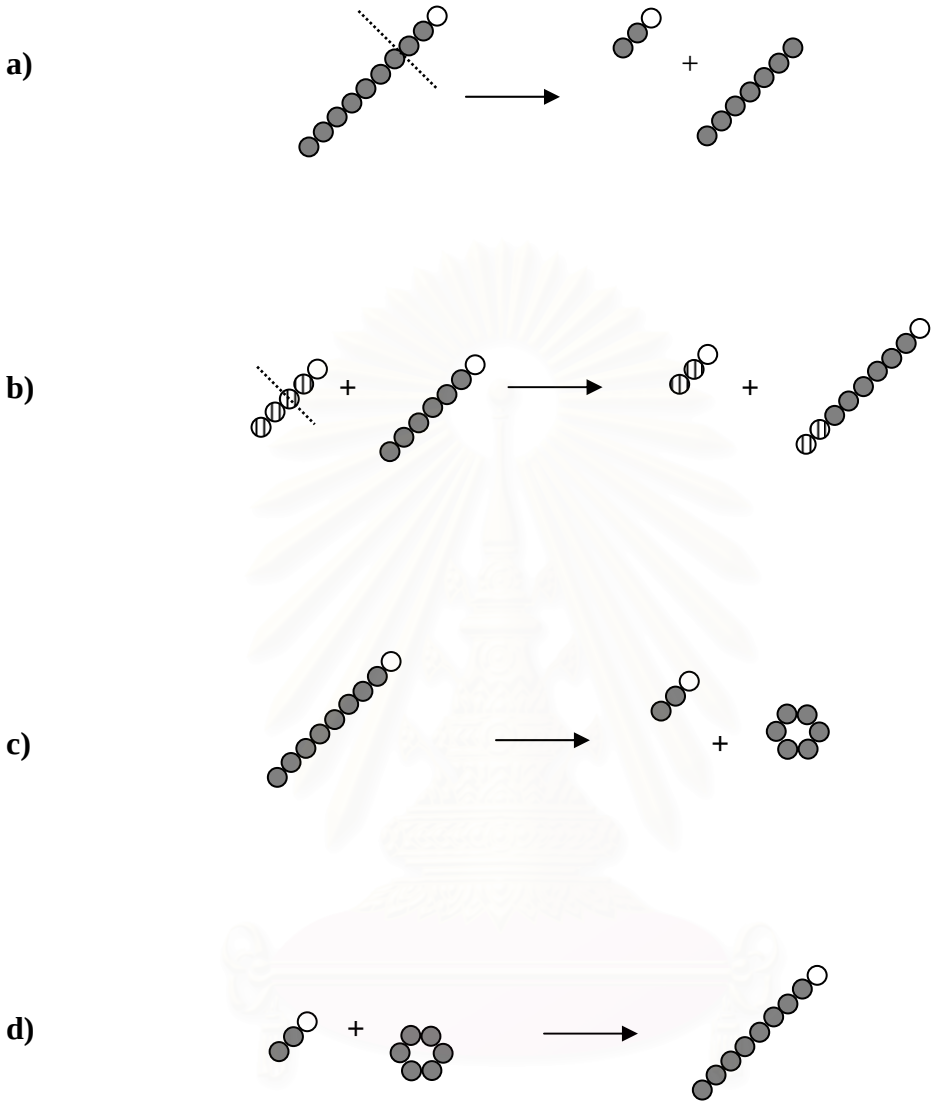

Figure 5 Schematic representation of the CGTase catalyzed reactions

The circles represent glucose residues; the white circles indicate the reducing end sugars. (a) hydrolysis, (b) disproportionation, (c) cyclization, (d) coupling (Van der Veen *et al.*, 2000).

Table 6 Properties of some bacterial CGTases (modified from Tonkova, 1998)

Producer	Optimum pH	Optimum temperature (^{o}C)	Molecular mass	Main CD Produced	Reference
Bacillus macerans ATCC 8514	6.1-6.2	60	139,000	-	DePinto, 1968
Bacillus macerans IFO 3490	5.0-5.7	55	-	α-CD	Kitahara, 1974
Bacillus megaterium No5	5.0-5.7	55	-	β-CD	Kitahara, 1974
Bacillus circulans var. *alkalophilus* ATCC 21783	4.5-4.7	45	88,000	β -CD	Nakamura, 1977
Bacillus stearothermophilus	6.0	-	68,000	α –CD	Kitahata, 1982
Klebsiella pneumoniae M5 al	6.0-7.2	-	68,000	α –CD	Bender, 1982
Bacillus fermus/lentus	6.0-8.0	50	75,000	γ-CD	Englbrecht, 1990
Bacillus sp. AL-6 (alkalophilic strain)	7.0-10.0	60	74,000	γ-CD	Fujita, 1990
Bacillus cereus NCIMB	5.0	40	-	α -CD	Jamuna, 1993
Bacillus halophilus INMIA 3849	7.0	60-62	71,000	β -CD	Abelian, 1995
Thermoanaerobacterium thermosulfurigene EM1	4.5-7.0	80-85	68,000	β -CD	Wind, 1995
Paenibacillus sp. A11	6.0-7.0	40-60	72,000	β –CD	Kaskangam, 1998
Paenibacillus sp. RB01	6.0	55	65,000	β –CD	Tesana, 2001

- no data

1.4.1 Applications of transglucosylation activity of CGTase

The transglucosylation activity of CGTase is currently utilized for industrial application in saccharides and glycosides products such as coupling sugar and transglycosylated stevioside. Coupling sugar is a commercial name of the maltooligosyl-fructose. Using the intermolecular transglycosylation reaction of CGTase, the coupling sugar is produced from the mixture of starch hydrolyzates as substrate and sucrose as an acceptor (Kitahata, 2000). The maltosyl fructose is initially formed and also acts as acceptor, yielding maltotriosyl fructose. A series of maltooligosyl fructosides is subsequently formed (Martín *et al*., 2003). The industrial processes for the CGTase catalyzed synthesis of coupling sugar have been patented using the enzyme from *Bacillus macerans* and *Thermoanaerobacter* sp. (Peterson, 1992). The sweetness of coupling sugar is between 55 and 65 percent of that of sucrose with the low cariogenicity property. It is widely employed in the manufacture of candies, cookies, chocolates etc. (Ikeda, 2007). The transglucosylation of stevioside, a plant flavonoid, using starch as a donor is also catalysed by CGTase. The enzyme from *Bacillus stearothermophilus* FERM-P No2222 (U.S.Pat.No.4,219,571) (Magomet et al., 2005) is used in industrial production of glucosyl stevioside. The glucosyl stevioside as the natural sweetener with a high-yielding and low cost production is now sold competitively on a mass commercial scale (Stevian Biotechnology, 2007).

Several works on transglycosylation of other interesting compounds to make useful glucosides or oligosaccharides by the action of CGTase have been conducted. These are in the research or in the development stage. Examples are as follows:

- In 1991, Agar and his colleagues synthesized 2-*0*-α-D-glucopyranosyl L-ascorbic acid (AA-2G) by transglucosylation of ascorbic acid by CGTase from *Bacillus circulans* and α-CD was used as a glucosyl donor.

- In 1994, Kometani and his colleagues synthesized 4^{G}-α-D-glucopyranosyl hesperidin by transglucosylation with CGTase from alkalophilic *Bacillus* species using soluble starch as a glucosyl donor. The solubility of mono

and diglucoside in water was about 300 times higher than that of hesperidin. The products also had a stabilizing effect on yellow pigment crocin, from fruits of *Gardenia jasminoides*, against ultraviolet irradiation.

- In 1995, Kometani and his colleagues synthesized 3^{G}-α-D-glucopyranosyl neohesperidin by transglucosylation of neohesperidin by CGTase from alkalophilic *Bacillus* species using soluble starch and beta-CD as a glucosyl donor. The solubility of neohesperidin monoglucoside in water was about 1500 times higher than that of neohesperidin, and the bitterness of product was about 10 times less than that of neohesperidin.

- In 2001, Martin and his colleagues synthesized maltooligosaccharides (MOS) (G_2-G_7) using *Thermoanaerobacter* sp. CGTase. Starch and glucose were used as a donor and an acceptor, respectively. The excellent yield of MOS (79%) was reported.

- In 2002, Campa and his colleagues synthesized a trisaccharide (Glcα1-4 Glcα1–6Glc) and a tetrasaccharide (Glcα1–4Glcα1–4Glcα1–6Glc), the structure of repeating unit of pullulan, by transglucosylation with CGTase from *Aspergillus niger*. α-Cyclodextrin and isomaltose was used as a donor and an accepter, respectively.

- In 2007, Prousoontorn and Pantatan synthesized 2-*O*-α-glucopyranosyl-L-ascorbic acid (AA-2G) by transglucosylation with immobilized CGTase from *Paenibacillus* sp. A11 and β-CD was used as a glucosyl donor. The yield of AA-2G was 2.92% and the immobilized CGTase retained its activity up to 74.4% of the initial catalytic activity after being used for 3 cycles.

1.5 Transglucosylation of catechins

Glucosylation is considered to be an important method for the structural modification of phenolic compounds with useful biological activities because it can increases water solubility and improves properties such as stability against oxidation,

light and heat. Furthermore, some enzymatic reaction which sugar molecules are attached to flavonoids using microbial glycosyltransferases generally proceed regioselectively and stereoselectively without protection and deprotection processes, and this facilitates anomer-selective glucosylation (Sato *et al*., 2000). The importance of transglucosylation reaction in synthesizing specific and useful products is well organized.

Although catechins have many potential applications, catechins powders are poorly soluble in water, bitter, brown, easily oxidized and degraded by light irradiation. Therefore, they have limitation to be used as a natural food additive or medicine (Ishizu *et al*., 1999). However, these problems can be overcome by modifying them with sugar molecules. Glucosylation of catechins through enzyme catalysis using various glycosyltransferases, sucrose phosphorylase, or glucansucrase has been studied. These works are summarized as follows.

- In 1993, Funayama and his colleagues synthesized (+)-catechin 3'-*O*-α-D-glucopyranoside by transglucosylation with CGTase from *Bacillus macerans* (α-CGTase) and starch was used as a glucosyl donor. HPLC analysis (peak area) of the reaction mixture suggested that 4.3 % of catechin was converted to glucoside. This glucoside inhibited the activity of tyrosinase from mushroom which can be applied to prevent discoloring of perishable foods or to suppress melanogenesis in animal cells.

- In 1993, Kitao and his colleagues synthesized (+)-catechin 3'-*O*-α-D-glucopyranoside by transglycosylation with sucrose phosphorylase from *Leuconostoc mesenteroid* and sucrose was used as a glucosyl donor. The browning resistance of glucoside to light irradiation was greatly increased and the solubility in water was 50-fold higher than of catechin. While antioxidative activity of glucoside with riboflavin was almost equal to that of catechin.

- In 1999, Meulenbeld and his colleagues synthesized (+)-catechin-4'-*O*-α-D- and catechin-4',7-*O*-α-di-D-glucopyranoside by transglucosylation with glucosyltransferase from *Streptococcus* sp. and sucrose was used as a

glucosyl donor. A maximum yield expressed as the ratio of moles of glucosides formed to moles of catechin initially added was 90%.

- In 2000, Sato and his colleagues synthesized (+)-catechin-3'-*O*-α-D-glucopyranoside by transglucosylation with glucosyltransferase from *Xanthomonas campestris* WU-9701 and maltose was used as a glucosyl donor. The maximum molar conversion yield was 57.1%. The solubility of the glucoside was 100-fold higher than of catechin. The glucoside had no bitter taste and had a slight sweet taste compared with catechin.

- In 2006, Moon and his colleagues synthesized epigallocatechin gallate 4''-mono, 7,4''-di-, and 4', 4''-di-*O*-α-D-glucopyranoside (EGCG-G1, EGCG-G2A, and EGCG-G2B) by transglucosylation with glucansucrase from *Leuconostoc mesenteroid* and sucrose was used as a glucosyl donor. The % yield (% molar ratio) of EGCG-G1, -G2A, and -G2B were 15.7%, 22.7%, and 23.8% of reacted EGCG, respectively. EGCG-G1 and EGCG-G2A were reported for the first time. The EGCG glucosides exhibited the reduced antioxidant effects, depending on their structures (EGCG > EGCG-G1 > EGCG-G2A > EGCG-G2B). They also uniformly exhibited greater browning resistance than was observed in EGCG while the water solubility of EGCG-G1, -G2A, and -G2B were 69, 126, and 122 times higher, respectively, than that of EGCG.

From previous studies on production of glucosides of catechins, the structure, the yield, and properties of glucosides obtained were depended on enzyme and the condition used. This research thus aims to use CGTase from *Paenibacillus* sp. RB01 (a β-CGTase) isolated from hot spring area in Ratchburi province of Thailand to enzymatically synthesize glucosides of catechins, especially those of epicatechin, in the hope to obtain products with improved properties beneficial for industrial use.

Objectives of this research

1. Preparation of partially purified CGTase from *Paenibacillus* sp. RB01
2. Synthesis of catechins glucosides and detection of the products
3. Determination of transglucosylation efficiency
4. Optimization of transglucosylation reaction
5. Larger scale preparation, isolation, and characterization of glucosides
6. Determination of physical and biological properties of products

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CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Autoclave: Model HA 30, Hirayama Manufacturing Cooperation, Japan

Autopipette: Nichipet EX, Nichiryo, Japan

Balance: AB204-S, Mettler Toledo, Switzerland

Balance: PB303-S, Mettler Toledo, Switzerland

Centrifuge, refrigerated: Model J-21C, Beckman Instrument Inc, USA

C18-reversed phase column: Inertsil, Shimadzu, Japan

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

Evaporator: BÜCHI Rotavapor R-200, Switzerland

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

Incubator: Haraeus, Germany

Incubator shaker: Controlled environment: Psyco-therm, New Brunswick Scientific Co., USA

Laminar flow: Model BVT-124, International Sciencetific Supply Co., USA

Lyophilizer: LYPH-LOCK, LABCONCO, USA

Mass spectrometer: MicrOTOF, Bruker, Germany

Nuclear Magnetic Resonance: Varian Gemini 400 MHz, Varian, USA

PLC plates: Silica gel 60 F_{254} 1 mm, Merck, Germany

pH meter: pH900, Precisa, Switzerland

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

UV-VIS Spectrophotometer: DU650 Spectrophotometer, Beckman, USA

VIS Spectrophotometer: 6400 Spectrophotometer Jenway, LABQUIP, England

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

Water bath: Memmert, Germany

2.2 Chemicals

Acrylamide: Merck, USA

Agar: Merck, Germany

Amyloglucosidase (Glucoamylase) from *Aspergillus niger*: Fluka, Switzerland

Bacto-peptone: Difco Laboratories, USA

Bovine serum albumin (BSA): Sigma, USA

Catechin: Sigma, USA

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

β-Cyclodextrin: Nihon shokuhin kako company Ltd., Japan

Dialysis tubing: Sigma, USA

2, 2-Diphenyl-1-picryhydrasyl (DPPH): Fluka, Switzerland

Epicatechin: Sigma, USA

Epigallocatechin gallate: Japan

Ethylenediamine tetraacetic acid (EDTA): Fluka, Switzerland

Ethyl acetate: Scharlau, Spain

Glacial acetic acid: BDH, England

Glycerol: Scharlau, Spain

D (+)-Glucose: Sigma, USA

α-Glucosidase from *S. cerevisiae*: Fluka, Switzerland

Glycine: Sigma, USA

Hydrochloric acid: Merck, Germany

Iodine: Baker chemical, USA

D (+)-maltose monohydrate: Fluka, Switzerland

2-Mercaptoethanol: Scharlau, Spain

Methanol: Merck, Germany

N, *N´*-Methylene-bis-acrylamide: Sigma, USA

Peptone from meat: Merck, USA

Phenolphthalein: BDH, England

Phosphoric acid: Merck, Germany

Potassium hydrogen phosphate: Merck, Germany

Sephadex LH-20: GE Healthcare, Sweden

Sodium acetate, Sodium carbonate: BDH, England

Sodium chloride: USB, USA

Sodium dodecyl sulfate: Sigma, USA

Di-Sodium hydrogenphosphate: Fluka, Switzerland

Sodium hydroxide: Carlo Erba, Italy

Soluble starch, potato: Sigma, USA

Standard molecular weight marker protein: Sigma, USA

Sulphuric acid: BDH, England

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

Tris (hydroxymethyl)-aminomethane: USB, USA

Yeast extract: Scharlau, Spain

2.3 Bacteria

Paenibacillus sp. RB01, isolated from hot spring area at Ratchaburi province, Thailand, was screened for CGTase activity (Tesana, 2001).

2.4 Media Preparation

2.4.1 Medium I

Medium I consisted of 0.5% beef extract, 1.0% peptone, 0.2% NaCl, 0.2% yeast extract and 1.0% soluble starch. The pH of medium was adjusted to 7.2 (Tesana, 2001). For solid medium, 1.5% agar was added.

2.4.2 Horikoshi's medium

Horikoshi's medium, slightly modified from Horikoshi (1971) by Rutchtorn, (1993), contained 1.0% soluble starch, 0.5% peptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_20$ and 0.75% Na_2CO_3. The pH of medium was adjusted to 10.1-10.2.

2.5 Cultivation of bacteria

2.5.1 Starter inoculum

Paenibacillus sp. RB01 was streaked on solid Medium I and incubated at 37 ºC for 18 hours. One loop was inoculated into liquid Medium I and grown until A_{660} reached 0.3-0.5.

2.5.2 Enzyme production

Starter inoculum of *Paenibacillus* sp. RB01 was 1.0% transferred into 300 ml of Horikoshi's medium and cultivated at 40 ºC for 72 hours. Cells were removed by centrifugation at 3,000 rpm at 4 ºC. Culture broth with crude CGTase was collected and kept at 4 ºC for further purification.

2.6 Partial purification of CGTase

CGTase from the culture broth was purified by starch adsorption method (Kato and Horikoshi, 1985, modified by Laloknam, 1997).

2.6.1 Starch adsorption

Corn starch was oven dried at 120ºC 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 5,000 rpm 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 5,000 rpm for 30 minutes. The enzyme solution was concentrated by aquasorb and was dialyzed against 50 mM phosphate buffer, pH 6.0 containing 10 mM $CaCl_2$ at 4 ºC.

2.7 Polyacrylamide Gel Electrophoresis (PAGE)

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

2.7.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The denaturing gel was carried out with 0.1% (w/v) SDS in 10.0% (w/v) separating and 5.0% (w/v) stacking gels with Tris-glycine buffer, pH 8.0 containing 0.1% SDS as electrode buffer (see Appendix 1). 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 2 hours. The slab gels were destained with a solution of 10% methanol and 10% 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 assay of starch degrading (dextrinizing) activity and cyclization (CD-forming) activity.

2.8.1 Dextrinizing activity

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

Sample (10-100 μl) was incubated with 0.3 ml starch substrate (0.2g% soluble potato starch in 0.2 M phosphate buffer, pH 6.0) at 40ºC for 10 minutes. The reaction was stopped with 4 ml of 0.2 M HCl. Then 0.5 ml of iodine reagent (0.02% I_2 in 0.2% KI) was added. The mixture was adjusted to a final volume of 10 ml with distilled water before measuring the absorbance at 600 nm. For a control tube, 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 the described conditions.

2.8.2 Cyclization activity assay

Cyclization activity was determined by the phenolphthalein method (modified from Goel and Nene (1995) by Yenpetch (2002)). Purified CGTase was added to 1.0 ml of 6.0% soluble starch in 0.2 M acetate buffer, pH 6.0. The reaction mixture was incubated for 30 minutes at 60 ^{o}C. Stop the reaction by boiling for 10 minutes. Fifty

microlitres of the reaction mixture was incubated with 2.0 ml of phenolphthalein solution. Absorption was measured at 550 nm and β-CD formed was calculated using the calibration curve of standard of β-CD phenolphthalein complex (see Appendix 3).

Phenolphthalein solution was prepared by mixing 1 ml of 4 mM phenolphthalein solution in absolute ethanol, 100 ml of 125 mM Na_2CO_3 solution in distilled water and 4 ml ethanol. This solution must be freshly prepared. β-CD standard in the range 0-2.5 mM was used to make calibration curve.

One unit of activity was defined as the amount of enzyme able to produce 1 μmole of β-CD per minute under the described condition.

2.9 Protein determination

Protein concentration was determined by Bradford's method (1976), using bovine serum albumin as the standard protein (see Appendix 3).

One hundred microlitres of sample was mixed with 1 ml of Coomassie blue reagent and left for 5 minutes before recording the absorbance at 595 nm. One litre of Coomassie blue reagent contained 100 mg of Coomassie blue G-250, 50 ml of 95% ethanol, 100 ml of 85% H_3PO_4 and distilled water.

2.10 Synthesis of catechins glucosides and detection of the products

2.10.1 Acceptor specificity

Glucosides of catechin (C), epicatechin (EC) and epigallocatechin gallate (EGCg) were prepared by incubation of partially purified CGTase (30 U/ml) in phosphate buffer, pH 6.0 with 1.8% beta-CD as glucosyl donor and 0.5% acceptor (C, EC and EGCg) at 40 °C for 24 hours. The products were analyzed by thin-layer chromatography (TLC).

2.10.2 Donor specificity

Transglucosylation efficiency was investigated by incubating 0.5% EC and CGTase (30 U/ml) in phosphate buffer, pH 6.0 with 1.8% different glucosyl donors: starch, beta-CD or maltoheptaose (G7) at 40 °C for 24 hours. The products were analyzed by TLC and HPLC, respectively.

2.10.3 Detection of products

2.10.3.1 Thin layer chromatography (TLC)

The products were analyzed by thin-layer chromatography (TLC) using silica gel 60. Solvent system was ethyl acetate–acetic acid-water (3:1:1). Spots were detected by spraying with H_2SO_4-methanol (1:2) (Funayama *et al*., 1993) followed by heating at 120 °C for 20 minutes.

2.10.3.2 High performance liquid chromatography (HPLC)

C18-Reverse phase HPLC column (4.6 x 250 mm) was used for quantitative analysis. As mobile phase, a 22:78 mixture of methanol and water was used at a constant flow rate of 0.5 ml/min at 35 °C. The products were detected by UV detector at 279 nm.

2.10.4 Determination of transglucosylation efficiency

Efficiency was judged by transglucosylation yield, which could be determined by two methods. The first method was to calculate from the ratio of disappearance of the amount of epicatechin acceptor to the initial amount (Park *et al*., 1998) as the equation:

$$\text{Transglucosylation yield (\%)} = \frac{\text{Amount of EC disappeared}}{\text{Amount of EC at } t_o} \times 100$$

The second method was to measure the product yield directly. The yield of transglucosylated products was calculated from peak area of product to that

of initial EC concentration in HPLC profile, using the equation:

$$\text{Product yield (\%)} = \frac{\text{Amount of product}}{\text{Amount of EC at } t_o} \times 100$$

2.11 Preliminary characterization of glucoside products

To preliminary investigate number of glucosyl residues attached to EC, glucoamylase (20 U/ml) from *A. niger* was added to the reaction mixture after 24 hours incubation, then the mixture was further incubated at 40 °C for 1 hour. And to confirm linkage between EC and glucosyl unit, α-glucosidase (20 U/ml) from *S.cerevisiae* was added to the reaction mixture after glucoamylase treatment and further incubated at 40 °C for another 3 hours. The products after treatment with the two enzymes were analyzed by TLC.

2.12 Optimization of transglucosylation reaction

2.12.1 Effect of pH

The reaction was performed by incubation of CGTase (30 U/ml) with 0.5% (w/v) epicatechin and 1.8% (w/v) beta-CD in 10 mM buffer in the pH range of 5.0 to 8.0 at 40 °C for 24 hours. The buffers used were sodium acetate (pH 5.0-6.0), potassium phosphate (pH 6.0-7.0) and Tris-HCl (pH 7.0-8.0) (see Appendix 2). The reaction was stopped by boiling for 5 minutes, and then analyzed by HPLC. The optimum pH was judged from the determination of percent yield of transglucosylated products.

2.12.2 Effect of enzyme concentration

The reaction was performed by incubation of CGTase at various enzyme concentrations (20, 40, 60, 80, 100, and 120 U/ml) with 0.5% (w/v) epicatechin and 1.8% (w/v) beta-CD in the buffer at optimum pH at 40 °C for 24 hours. The reaction was stopped by boiling for 5 minutes, and then analyzed by HPLC. The optimum

enzyme concentration was judged from the determination of percent yield of transglucosylated products.

2.12.3 Effect of epicatechin concentration

The reaction was performed by incubation of appropriate concentration of CGTase with various epicatechin concentrations (0.06, 0.125, 0.25, 0.50, 1.0, and 2.0 %, w/v) and 1.8% (w/v) beta-CD in the buffer at optimum pH at 40 °C for 24 hours. The reaction was stopped by boiling for 5 minutes, and then analyzed by HPLC. The optimum epicatechin concentration was judged from the determination of percent yield of transglucosylated products.

2.12.4 Effect of beta-CD concentration

The reaction was performed by incubation of appropriate concentration of CGTase with various beta-CD concentrations (0.125, 0.25, 0.50, 1.0, and 1.8%, w/v) and optimum epicatechin concentration in the buffer at optimum pH at 40 °C for 24 hours. The reaction was stopped by boiling for 5 minutes, and then analyzed by HPLC. The optimum beta-CD concentration was judged from the determination of percent yield of transglucosylated products.

2.12.5 Effect of temperature

The reaction was performed by incubation of appropriate concentration of CGTase with optimum concentrations of epicatechin and beta-CD donor in the buffer at optimum pH at various temperatures (30, 40, 50, 60 and 70 °C) for 24 hours. The reaction was stopped by boiling for 5 minutes, and then analyzed by HPLC. The optimum temperature was judged from the determination of percent yield of transglucosylated products.

2.12.6 Effect of incubation time

The reaction was performed by incubation of appropriate concentration

CGTase with optimum concentrations of epicatechin and beta-CD at optimum pH and temperature for various times (12, 24, 36, 48, and 60 hours). The reaction was stopped by boiling for 5 minutes, and then analyzed by HPLC. The optimum incubation time was judged from the determination of percent yield of transglucosylated products.

2.13 Larger scale preparation and isolation of glucosylated products

In the initial experiments: to prepare glucosides and to determine transglucosylation efficiency (2.10) and to optimize the transglucosylation reaction (2.12), small scale reaction mixture of 1 ml was used. To prepare higher amount of products for characterization, larger scale preparation (50 ml) of reaction mixture using optimum condition for transglucosylation as obtained from section 2.12 was performed. After the reaction, the mixture was concentrated by reducing the volume to 5 ml at 45 $^{\circ}$C using a rotary evaporator, and then applied on Sephadex LH-20 column (2.5 x 55 cm) equilibrated with distilled water. Then glucoside products were separated from sugars and enzyme by elution of the column with distilled water (2 L, 0.5 ml/min, fraction size 2 ml). Then epicatechin was eluted using 50% methanol. The fractions containing epicatechin glucosides were collected and concentrated with a rotary evaporator. The concentrated fractions of epicatechin glucosides were separated by HPLC as described in section 2.10. Each glucoside peak was collected for further characterization.

2.14 Characterization of epicatechin glucosides

2.14.1 Mass Spectrometry

Mass analysis of major glucoside 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.

2.14.2 Nuclear Magnetic Resonance

For structural elucidation, ^{1}H analysis was performed. ^{1}H NMR spectra were obtained in CD_3OD on a Varian Gemini 400 MHz spectrometer at the department of Chemistry, Chulalongkorn University.

2.15 Determination of properties of epicatechin glucosides

2.15.1 Solubility in water

Excess amounts of EC and EC glucosides were mixed with 200 μl of water in an eppendorf tube. After vortexing for 5 minutes at room temperature, each sample was incubated at 30 °C for 15 minutes. The samples were filtrated through a 0.45 μm membrane for HPLC analysis, in order to determine the soluble concentrations.

2.15.2 Browning resistance

Browning resistance was examined in water solution (1.0 ml) containing 0.1% (w/v) of either EC or EC glucosides. The samples were then exposed to UV irradiation from the UV lamp (254 nm) at a distance of 35 cm for 10 hours. The absorbance at 460 nm of the solutions were then determined (modified from Moon *et al*., 2006).

2.15.3 Antioxidant activity

The antioxidant activity of EC and EC glucosides was evaluated using DPPH radical scavenging reaction (Abe *et al.,* 2000). Each of the samples (10, 12.5, 25, 50, 100, 200 μM) was dissolved in ethanol (30 μl) and mixed thoroughly with a 100 μM DPPH in ethanol solution (270 μl). After 10 minutes in the dark, at room temperature, the absorbance of the mixture was measured at 517 nm. DPPH radical scavenging activity was then evaluated by analyzing the percentage of decrease in the absorbance of the sample compared to a blank (ethanol). The IC_{50} value designates the inhibitory concentration at which the absorbance was reduced by 50%.

CHAPTER III

RESULTS

3.1 Partial purification of CGTase

After cultivation of bacteria *Paenibacillus* sp. RB01 in Horikoshi's medium for 60 hours, the cells were removed by centrifugation. The crude CGTase in the culture filtrate was partially purified by starch adsorption method and was then concentrated by aquasorb and dialyzed against 20 mM phosphate buffer, pH 6.0. The enzyme activity was determined by dextrinizing assay. The purification fold and recovery of CGTase obtained at each step are shown in Table 7. The purification of enzyme was increased by 14.6 fold and a 69.8 percent yield was obtained.

The purity of partially purified CGTase was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After starch adsorption step, the intensity of CGTase band of molecular weight approximately 65 kDa (Yenpetch, 2002) was significantly increased and the band was about 2/3 of total proteins (Figure 6, lane 3).

3.2 Synthesis of catechins glucosides and detection of the products

3.2.1 Acceptor specificity

Acceptor specificity was determined by varying the types of catechin used. After incubation of CGTase (30 U/ml) with 1.8% beta-CD and 0.5% catechins acceptor (catechin, C; epicatechin, EC; and epigallocatechin gallate, EGCg) at 40 $^{\circ}$C for 24 hours, reaction products were analyzed by TLC (Figure 7). The migration of standard catechins in this TLC system was in the order of C >EC >EGCg (lane a, b, and c). At initial time of incubation, the only spots of catechins and beta-CD used as substrate were found (lane f, h and j). Catechins were migrated closed to the solvent front; however, beta-CD slightly moved from the origin. After 24 hours of incubation, the spots expected to be glucosylated catechins were observed (lane g, i

Table 7 Partial purification of CGTase from *Paenibacillus* sp. RB01

Step	Volume (ml)	Activity* (U/ml)	Protein (mg/ml)	Sp. activity (U/mg protein)	Purifi--cation (fold)	Total activity ($Ux10^3$)	Yield (%)
crude enzyme	2,000	53.1	0.31	171.3	1	106	100
starch adsorption	47	1579.7	0.63	2507.5	14.6	74.2	69.8

* Dextrinizing activity

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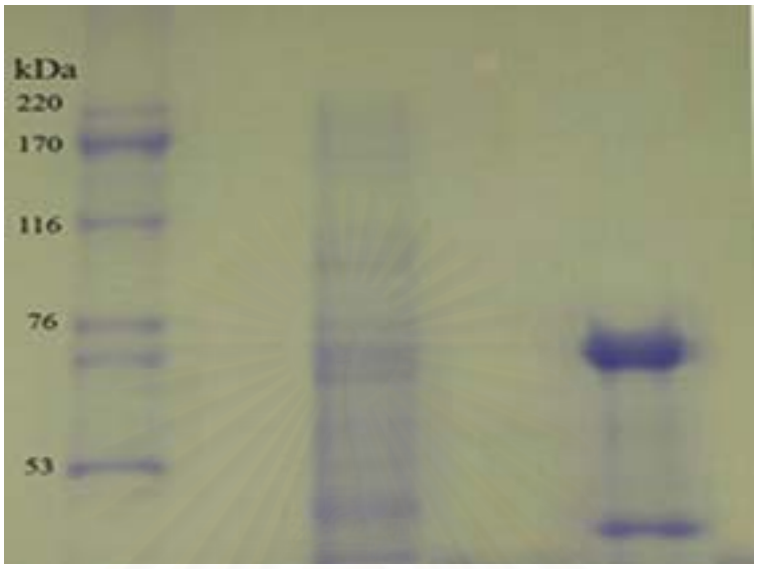

Figure 6 SDS-PAGE of crude and partially purified CGTase

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

Lane 2: Crude enzyme (50 μg)

Lane 3: Starch adsorbed enzyme (20 μg)

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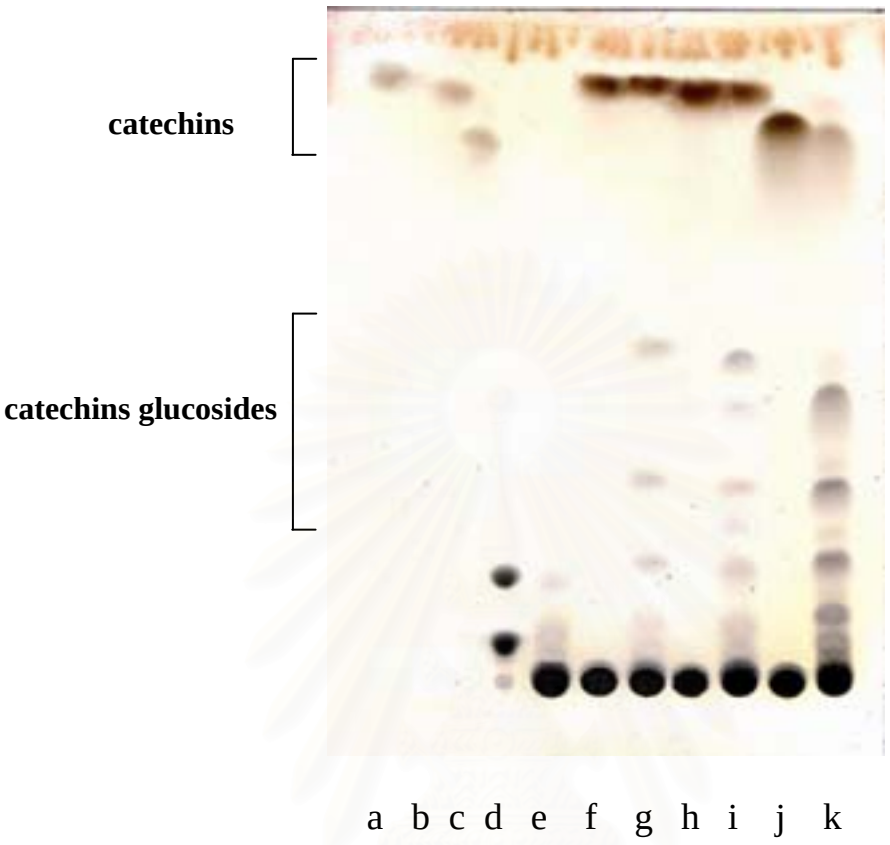

Figure 7 TLC chromatogram of reaction products of CGTase incubated with catechins and beta-CD (conditions were: 0.5% catechins, 1.8% beta-CD, 30 U/ml CGTase, pH 6.0, 40 °C)

(a) standard catechin (5 µg)
(b) standard epicatechin (5 µg)
(c) standard epigallocatechin gallate (5 µg)
(d) standard glucose and maltose (15 µg each)
(e) control reaction (beta-CD + CGTase, no acceptor)
(f)-(g) reaction mixture with catechin, 0 h and 24 h
(h)-(i) reaction mixture with epicatechin, 0 h and 24 h
(j)-(k) reaction mixture with epigallocatechin gallate, 0 h and 24 h

Table 8 Rf values from TLC analysis of standard catechins, saccharides and the reaction products

Standard/Product			Rf
Standards	Saccharides	Glucose	0.20
		Maltose	0.10
		Beta-CD	0.01
	Catechins	C	0.92
		EC	0.90
		EGCg	0.86
Reaction products top ↓ bottom	From C acceptor		
		I	0.56
		II	0.40
		III	0.24
	From EC acceptor		
		I	0.55
		II	0.48
		III	0.38
		IV	0.32
	From EGCg acceptor		
		I	0.50
		II	0.37
		III	0.25

Table 9 Acceptor specificity of CGTase towards catechins

Acceptor (number of hydroxyl groups)	Glucosylated products
(+) catechin (5)	++
(-) epicatechin (5)	++
(-) epigallocatechin gallate (8)	+++

The intensity of spots of major products (by eye-observed) were: +++, high; ++, medium

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and k). The transfer products of catechins were distinguished from glucose and maltose (lane d) by their larger Rf values. In the control condition, when only CGTase and beta-CD were incubated (lane e), the reaction products were oligosaccharides which run slower than those glucosides of catechins. The Rf values of all standards and products were presented in Table 8. When different catechins were used as acceptor, EC gave at least two major and two minor transfer glucosylated products (lane i) while C and EGCg gave at least three major products (lane g and k). The spot intensity of the products using EGCg as an acceptor was stronger than those of EC and C, respectively. The degree of intensity difference was estimated as shown in Table 9. The result thus suggested that EGCg showed higher acceptor specificity to CGTase from *Paenibacillus* sp. RB01. However, synthesis and characterization of glucosides of EGCg have been recently reported while no previous characterization of EC glucosides has been reported. Also from spot intensity of the products, the estimated amounts of EC glucosides were not much less than EGCg glucosides, EC was then chosen as appropriate acceptor in this work.

3.2.2 Donor specificity

In order to elucidate donor specificity, 0.5% EC was used as acceptor while 1.8% starch, beta-CD, or G7 were used as donor in transglucosylation reaction with CGTase (30 U/ml). Incubation was performed at 40 °C for 24 hours and the synthesized products were detected by TLC.

From TLC chromatogram (Figure 8), all of the reaction mixtures using the three donors gave the same two major (named Glu I and Glu II) and two minor glucoside products. While the intensities of the glucoside spots were similar and could not be distinguished by eye-observed. Transglucosylation efficiency of different donors was also investigated by HPLC using the condition as described in section 2.10. The transglucosylation yield was determined from the ratio of disappearance of the amount of epicatechin acceptor to the initial amount. When starch, beta-CD, and maltoheptaose were used as a donor, the detected transglucosylation yields were 22.6, 22.4, and 21.4 %, respectively.

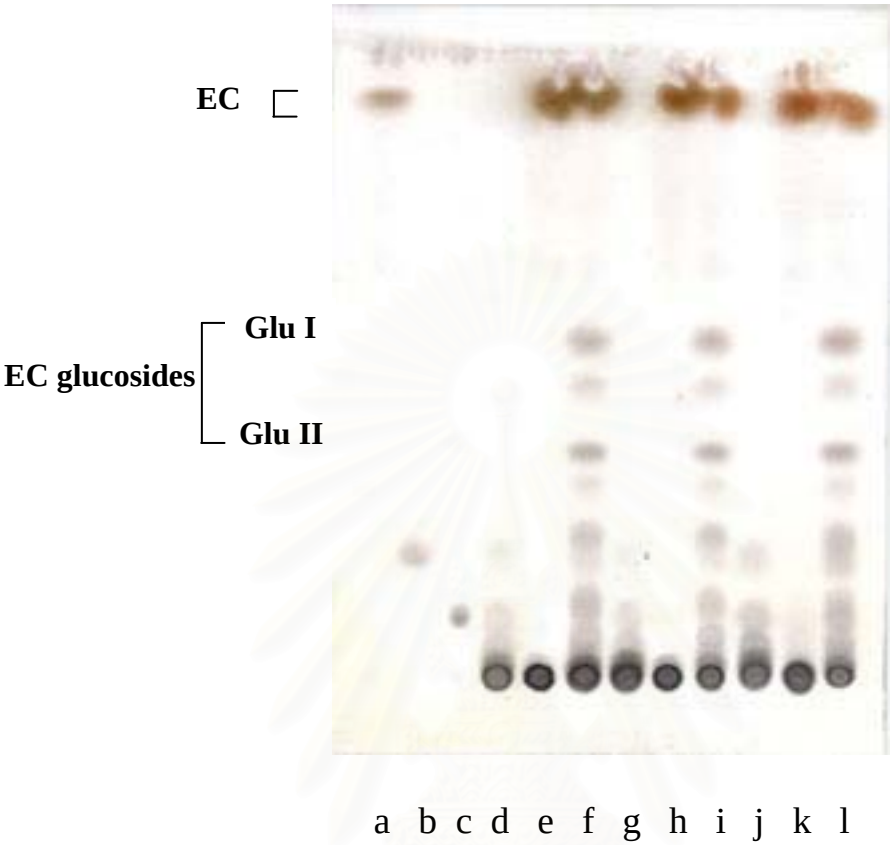


Figure 8 TLC chromatogram of reaction products of CGTase incubated with epicatechin and various donors (conditions were as in Figure 7)

(a) standard epicatechin (15 µg)
(b) standard glucose (10 µg)
(c) standard maltose (10 µg)
(d) control reaction (starch + CGTase)
(e)-(f) reaction, starch as a donor, 0 and 24 h, respectively
(g) control reaction (beta-CD + CGTase)
(h)-(i) reaction, beta-CD as a donor, 0 and 24 h, respectively
(j) control reaction (maltoheptaose + CGTase)
(k)-(l) reaction, maltoheptaose as a donor, 0 and 24 h, respectively.

3.2.3 Preliminary characterization of EC glucoside products

To preliminary characterize EC glucoside products, the number of glucosyl units and the configuration of the products were investigated. The enzyme glucoamylase (20 U/ml) from *A. niger* was added to the reaction mixture after 24 hours incubation, then further incubated at 40 °C for 1 hour and analyzed by TLC (Figure 9). The results showed that prior to glucoamylase treatment, two major glucoside products, "Glu I" (the upper spot) and "Glu II" (the lower spot) (lane c) were observed. After treated with glucoamylase, Glu II was disappeared. While the intensities of Glu I and glucose spot were increased (lane d). The result suggested that Glu I and Glu II were EC monoglucoside and oligoglucoside, respectively. To further hydrolyze the glucoside products, α-glucosidase (20 U/ml) from *S. cerevisiae* was added to the reaction mixture after glucosidase and further incubated at 40 °C for 1 hour. No glucoside spot was found whereas the intensities of epicatechin and glucose spots were increased (lane e). However, the Glu II still occurred in the reaction mixture treated only with α-glucosidase while the Glu I spot was disappeared (lane f).

To further characterize the glucoside products, the reaction mixture was analyzed by HPLC using C18-reversed phase column with the condition as described in the section 2.10 (Figure 10). From HPLC profile, when reaction mixture (EC + CGTase + beta-CD) at time zero was analyzed, the peaks of catechin and epicatechin were detected at Rt 19 and 38 minutes, respectively (Figure 10A). After 24 hour incubation, the peak area of epicatechin was decreased while the four peaks of epicatechin glucosides were detected at Rt 27, 30, 45, and 48 minutes (Figure 10B). After treated with glucoamylase, Rt 27 and 45 minutes were disappeared (Figure 10C). If the reaction was further treated with α-glucosidase, Rt 30 and 48 minutes were also disappeared (Figure 10D).

To indicate that the HPLC peaks at Rt 27, 30, 45, 48 minutes corresponded to which spots in TLC, the fractions from these peaks were collected by HPLC fraction collector and then analyzed by TLC (Figure 11). From TLC chromatogram, the two major products with Rt 48 minutes had Rf equal to Glu I and that with Rt 45 minutes

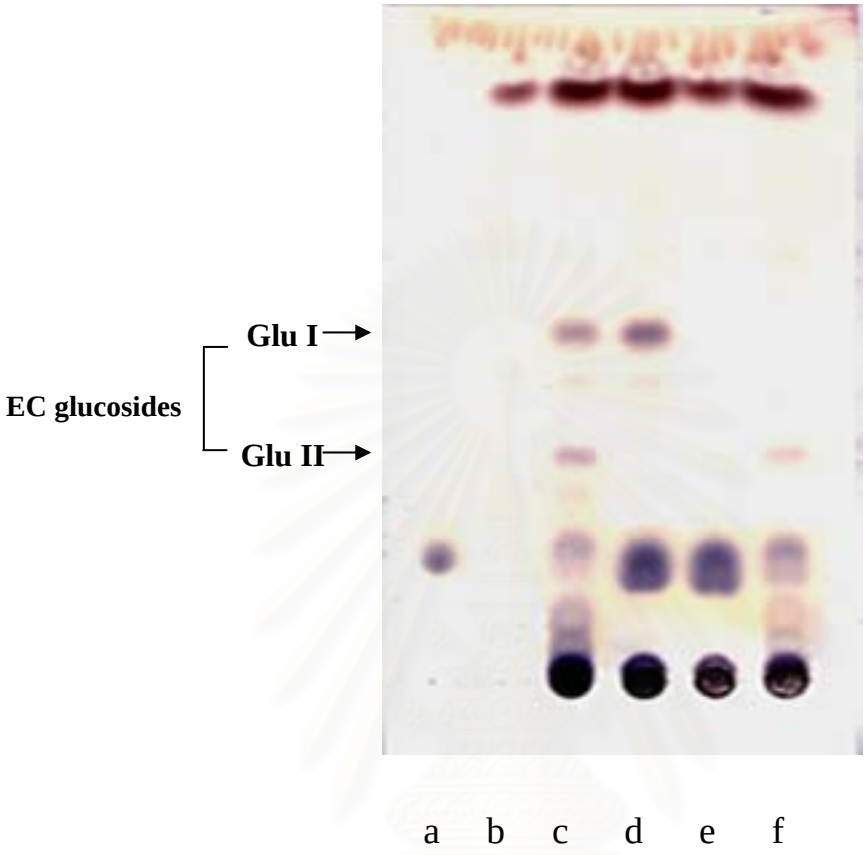
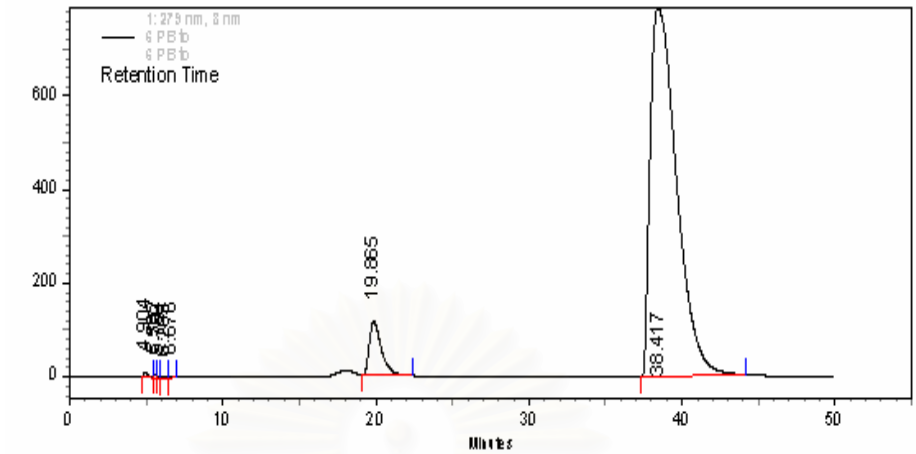


Figure 9 TLC chromatogram of reaction products of CGTase incubated with EC and beta-CD for 24 h (conditions were as in Figure 7)

a) standard glucose (15 µg)
b) standard EC (10 µg)
c) reaction mixture, 24 h (18 µl)
d) reaction mixture, 24 h with glucoamylase (18 µl)
e) reaction mixture, 24 h with glucoamylase and α-glucosidase (18 µl)
f) reaction mixture, 24 h with α-glucosidase (18 µl)

A.



B.

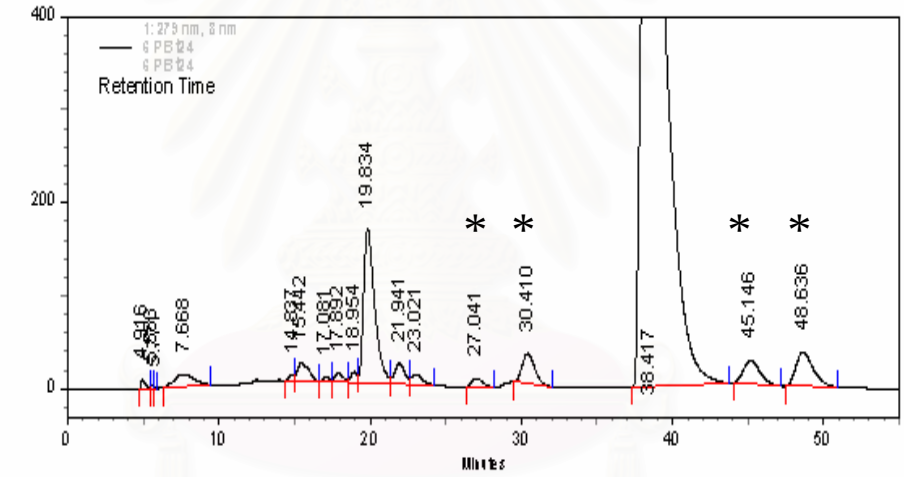


Figure 10 HPLC chromatogram of reaction products of CGTase with EC and beta-CD (conditions were as in Figure 7)

A. 0 h

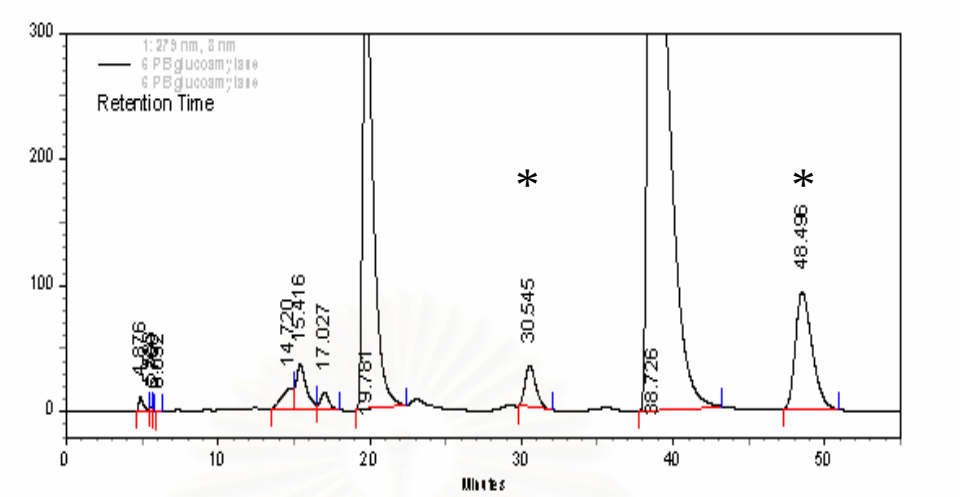
B. 24 h

Rt 19.8 - catechin

Rt 38.4 - epicatechin

* Rt 27.0, 30.4, 45.1, 48.6 - epicatechin glucosides

C.



D.

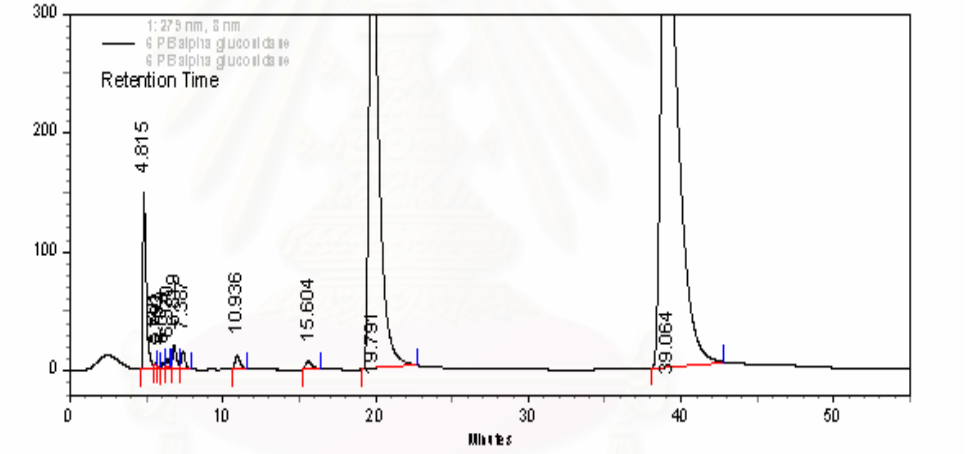


Figure 10 HPLC chromatogram of reaction products of CGTase with epicatechin and beta-CD (conditions were as in Figure 7) (continued)

C. 24 h, then treated with glucoamylase

D. 24 h, then treated with glucoamylase, followed by α-glucosidase

a b c d e

Figure 11 TLC chromatogram of EC glucoside products separated by HPLC (from figure 10B)

a) reaction mixture after 24 h incubation (8 µl)

b) product at Rt 27 min (15 µl)

c) product at Rt 30 min (15 µl)

d) product at Rt 45 min (15 µl) (Glu II)

e) product at Rt 48 min (15 µl) (Glu I)

had Rf equal to Glu II. While the two minor peaks at Rt 30 and 27 minutes had a little lower Rf values than those of Glu I and Glu II, respectively. The spots of peaks at Rt 27 and 30 min, however, were different from spots of the two minor products detected by TLC. From these results, it can be concluded that the products with Rt 27, 30, 45 and 48 min were EC glucoside products.

3.3 Optimization of transglucosylation reaction

The suitable conditions for the production of epicatechin glucosides were determined using beta-CD as a glucosyl donor. The reaction was performed as described in section 2.12 and reaction products were analyzed by HPLC. The optimum conditions were considered from the maximum percent yield of transglucosylated products, calculated from peak area of products to initial amount of epicatechin (see section 2.10.4).

3.3.1 Effect of pH

When incubating CGTase (30 U/ml) with 0.5% epicatechin and 1.8% beta-CD at various pH, at 40 $^{\circ}$C for 24 hours, it was found that product yields varied with pH. The percent yields of each transglucosylated product and total product yields were shown in Table 10 and Figure 12. The maximum total transglucosylation yield was obtained with 9.01% when the reaction mixture was performed in acetate buffer at pH 6.0. However, the total yields of products in the buffer at pH 6.0 (acetate buffer) and 7.0 (Tris-HCl buffer) were not significantly different. When phosphate buffer was used, the product yields were decreased at both pH 6.0 and 7.0. It was observed that the yield of each product was also maximum at these pHs. The relative yield of each product was in the order of Rt 48 min > Rt 30 min ≈ Rt 45 min >> Rt 27 min. In addition to the four products at Rt 27, 30, 45, and 48 min, a new product was observed at Rt 28 min. However, this product occurred only at pH 8.0.

During optimization by varying pH, the epimeric conversion from epicatechin to catechin was observed (Figure 13). From HPLC profile, in the reaction mixture of phosphate buffer, pH 6.0, the peak area of catechin at time zero was about 6% of total

Table 10 Percent yields of transglucosylated products at different pH

Buffer	pH	Product yield (%)					Total yield (%)
		Rt 27	Rt 28	Rt 30	Rt 45	Rt 48	
Acetate buffer	5.0	0.35	-	1.87	1.77	2.78	6.77
	6.0	0.55	-	2.43	2.38	3.65	9.01
Phosphate buffer	6.0	0.47	-	2.01	2.04	3.13	7.65
	7.0	0.57	-	1.93	1.90	2.87	7.27
Tris-HCl buffer	7.0	0.52	-	2.37	2.36	3.58	8.83
	8.0	0.43	0.69	2.11	1.71	2.66	7.60

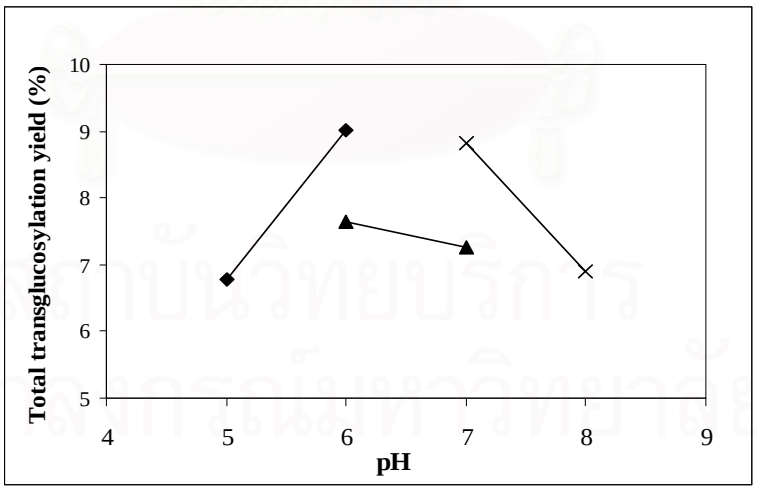


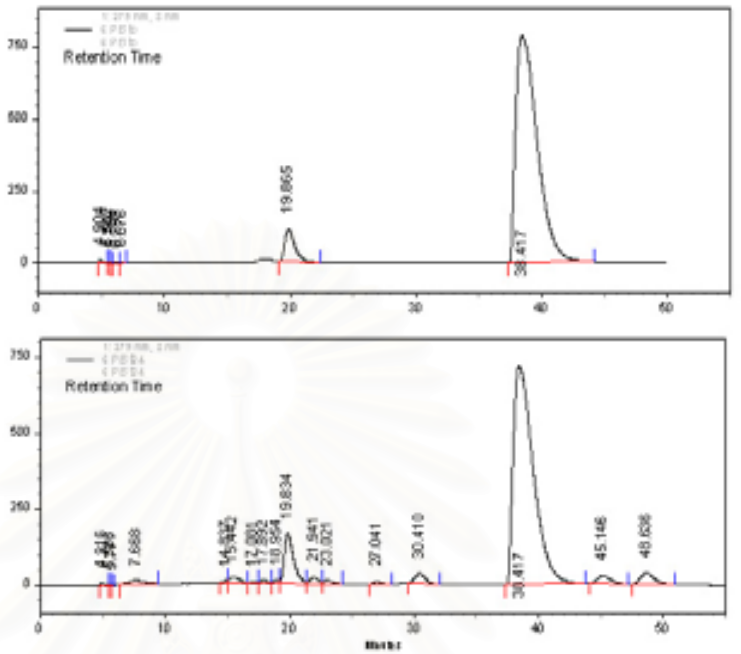
Figure 12 Effect of pH on transglucosylation yield

(◆) acetate buffer, (▲) phosphate buffer, (×) Tris-HCl buffer

A) Phosphate buffer pH 6.0

(1) 0 h

(2) 24 h



B) Tris-HCl buffer pH 8.0

(1) 0 h

(2) 24 h

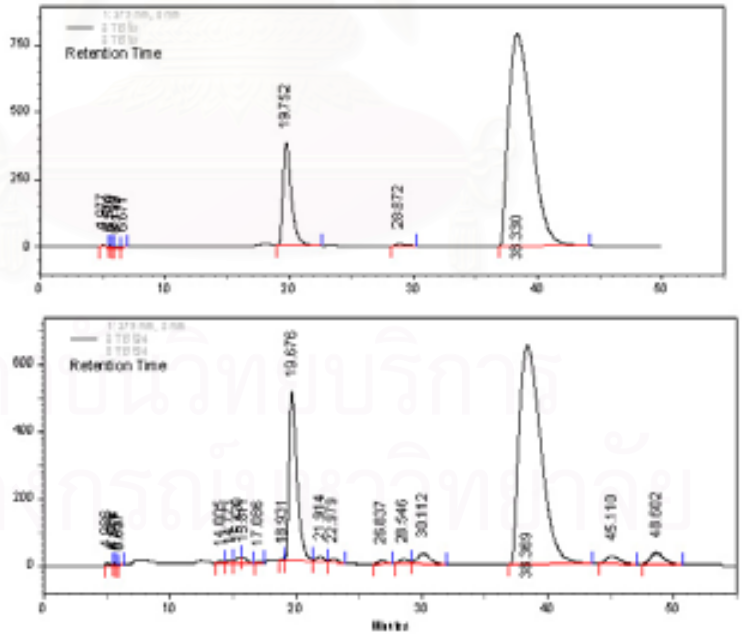


Figure 13 HPLC chromatogram of reaction products of CGTase with 0.5% EC and 1.8% beta-CD in buffer of different pH

A) pH 6.0, 0 and 24 h B) pH 8.0, 0 and 24 h

Table 11 The amount and the ratio of catechin to epicatechin in reaction mixtures at pH 6.0 and 8.0

Condition		Catechin* (%)	Epicatechin* (%)	Catechin : Epicatechin
Acetate buffer, pH 6.0	0 h	6.1	93.4	1 : 15
	24 h	7.3	79.3	1 : 10.9
Tris-HCl buffer, pH 8.0	0 h	14.8	84.5	1 : 5.7
	24 h	19.7	70.8	1 : 3.5

* The amount was determined from peak area of each compound compared to total peak area

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area (Figure 13A.1) and it was increased to 8% after 24 hours of incubation (Figure 13A.2). While the peak area of catechin in the Tris-HCl buffer, pH 8.0 at time zero was 15% (Figure 13B.1) and then increased to 20% after 24 hours of incubation (Figure 13B.2). This suggested that the epimerization of epicatechin to catechin occurred and was increased at alkaline pH (Table 11).

3.3.2 Effect of enzyme concentration

Using the conditions as mentioned in 3.3.1 at pH 6.0 with variable concentrations of CGTase, it was found that product yields significant increased at high concentration of CGTase. The percent yields of each transglucosylated product and total product yields were shown in Table 12 and Figure 14. The maximum total transglucosylation yield was obtained with 16.62% when the reaction mixture was incubated with 80 U/ml of CGTase. The yield of each product was also maximum at this CGTase concentration. Further increase in enzyme concentration did not result in increase in product yield. Yields significantly increased in the enzyme concentration range of 60-80 U/ml. The yield of each product was in the same order as the result in 3.3.1 but the relative amount was Rt 48 min >> Rt 30 min ≈ Rt 45 min >> Rt 27 min. The product at Rt 28 min was also detected with the very low yield (0.09%) at 80 U/ml of enzyme concentration.

3.3.3 Effect of epicatechin concentration

Using the conditions as mentioned in 3.3.2 with various epicatechin concentrations, it was found that product yields varied with epicatechin concentration. The percent yields of each transglucosylated product and total product yields were shown in Table 13 and Figure 15. The maximum total transglucosylation yield was obtained around 16.54-16.60% when the reaction mixture was incubated with epicatechin concentration in the range of 0.25-0.5%. By contrast, the yield of products was significantly decreased when epicatechin concentration was increased to 1.0%-2.0%. The 0.5% epicatechin concentration gave the maximum yield of the major product at Rt 48 min, thus it was chosen to be the optimum concentration. The yield of each product was still in the same order and relative amount as that found in 3.3.2.

Table 12 Percent yields of transglucosylated products at different CGTase concentrations

CGTase concentration (U/ml)	Product yield (%)					Total yield (%)
	Rt 27	Rt 28	Rt 30	Rt 45	Rt 48	
20	0.42	-	2.00	2.02	3.05	7.49
30	0.51	-	2.26	2.31	3.48	8.56
40	0.63	0.11	0.11	2.65	4.04	7.54
50	0.75	0.13	0.13	3.07	4.67	8.75
60	0.85	0.15	0.15	3.38	5.20	9.73
80	1.16	0.09	4.24	4.35	6.78	16.62
100	1.17	-	4.28	4.13	6.56	16.14
120	1.22	-	4.31	4.07	6.51	16.11

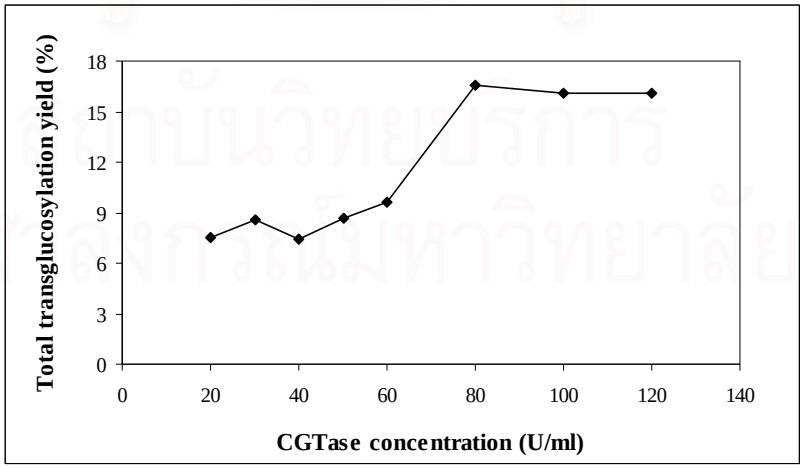


Figure 14 Effect of enzyme concentration on transglucosylation yield

Table 13 Percent yields of transglucosylated products at different epicatechin concentrations

Epicatechin concentration (%, w/v)	Product yield (%)					Total yield (%)
	Rt 27	Rt 28	Rt 30	Rt 45	Rt 48	
0.0625	1.10	-	4.51	4.13	6.06	15.80
0.125	1.12	-	4.76	4.19	6.27	16.34
0.25	1.20	-	4.57	4.34	6.49	16.60
0.50	1.15	-	4.26	4.35	6.78	16.54
1.0	0.92	0.78	3.92	3.68	6.07	14.60
2.0	0.40	-	2.04	1.97	3.64	8.05

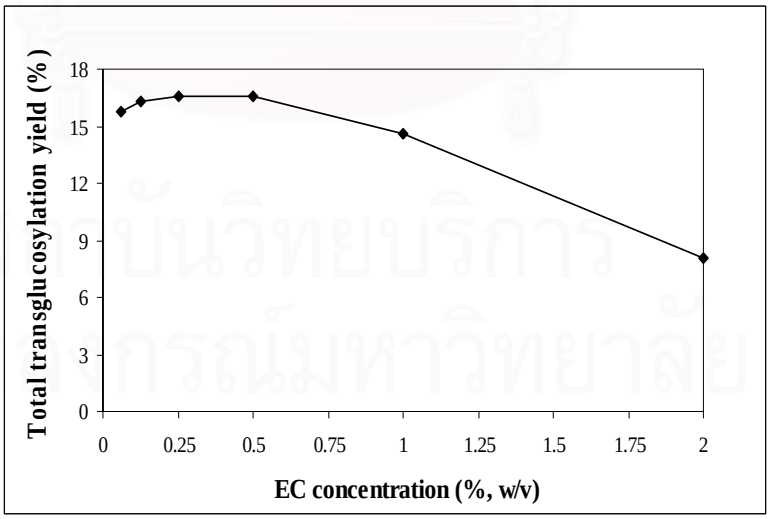


Figure 15 Effect of epicatechin concentration on transglucosylation yield

3.3.4 Effect of beta-CD concentration

Using the conditions as mentioned in 3.3.3 with various beta-CD concentrations, it was found that product yields varied with beta-CD concentration. The percent yields of each transglucosylated product and total product yields were shown in Table 14 and Figure 16. The maximum total transglucosylation yield was obtained at around 16.58-16.77% when the reaction mixture was incubated with beta-CD concentration in the range of 1.0 -1.8%. In order to save the use of beta-CD, 1.0% beta-CD was chosen to be the optimum concentration. The yield of each product at 1.0% beta-CD was still in the same order and relative amount as that found in 3.3.2.

3.3.5 Effect of temperature

Using the conditions as mentioned in 3.3.4 with various temperatures, it was found that product yields significantly varied with temperature. The percent yields of each transglucosylated product and total product yields were shown in Table 15 and Figure 17. The maximum total transglucosylation yield was obtained with 17.34% when the reaction mixture was incubated at 50 °C. It was observed that the yield of each product at 50 °C was in the order of Rt 48 min >> Rt 45 min > Rt 30 min >> Rt 27 min. The increase of temperature to 60-70 °C was obviously decreased the total yield. It was evident that at 70 °C, the maximum product at Rt 28 min was obtained with 2.63% yield while other products could not be observed.

During optimization by varying the temperature, the epimerization of epicatechin to catechin was also observed (Figure 18). From HPLC profile, at time zero of incubation, the peak area of catechin was about 8% of total area (Figure 18A). After incubation for 24 hours, the amount of catechin at 40 °C was about the same (Figure 18B). While at 70 °C, the catechin was increased to 44% (Figure 18C). This suggested that the epimerization was not only increased at alkaline pH but also at high temperature (Table 16).

Table 14 Percent yields of transglucosylated products at different beta-CD concentrations

Beta-CD concentration (%, w/v)	Product yield (%)					Total yield (%)
	Rt 27	Rt 28	Rt 30	Rt 45	Rt 48	
0.125	0.39	0.86	2.36	1.73	7.14	12.48
0.25	0.60	0.41	2.78	2.40	6.63	12.82
0.50	0.92	0.42	3.71	3.33	7.25	15.62
1.0	1.11	0.37	4.29	3.91	7.04	16.77
1.8	1.12	0.35	4.25	3.94	6.92	16.58

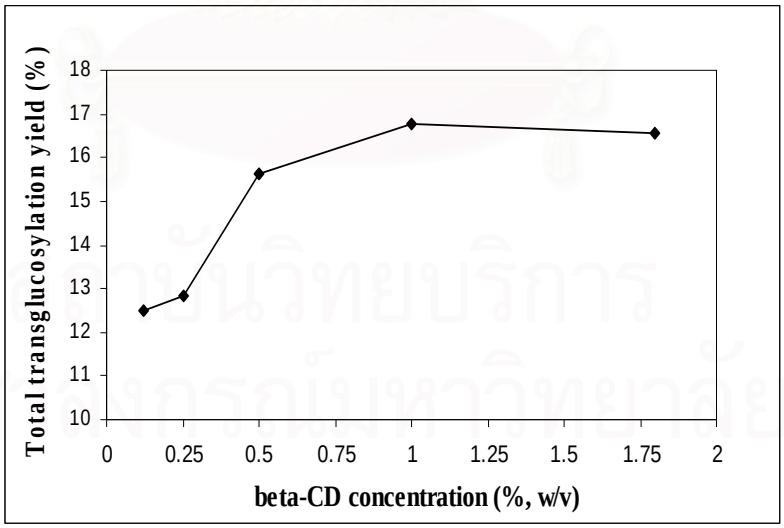


Figure 16 Effect of beta-CD concentration on transglucosylation yield

Table 15 Percent yields of transglucosylated products at different incubation temperatures

Temperature (°C)	Product yield (%)					Total yield (%)
	Rt 27	Rt 28	Rt 30	Rt 45	Rt 48	
30	0.73	-	3.35	3.22	5.63	12.93
40	1.00	-	3.90	3.61	7.61	16.12
50	1.19	0.63	3.33	4.50	7.69	17.34
60	0.83	1.22	3.01	2.33	4.39	11.78
70	-	2.63	-	-	-	2.63

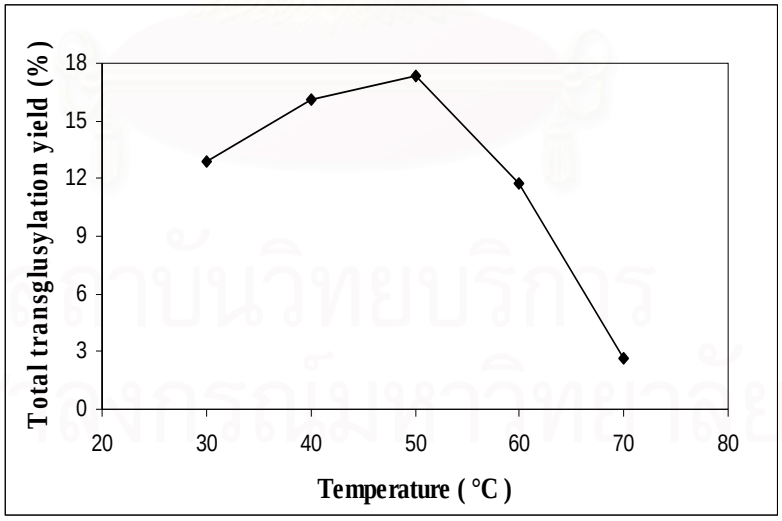
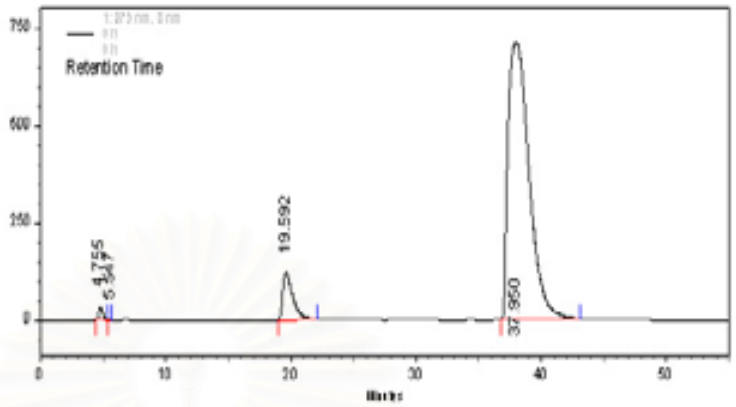
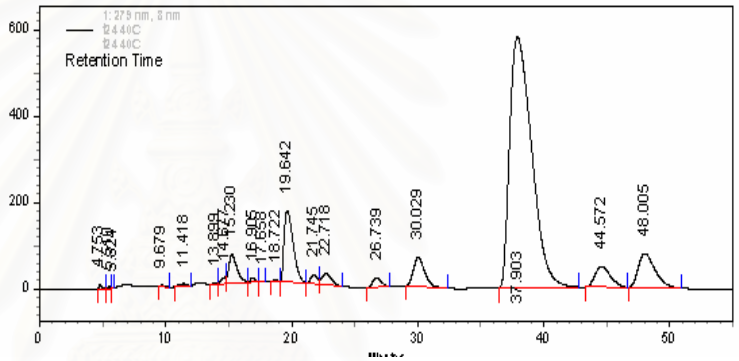


Figure 17 Effect of incubation temperature on transglucosylation yield

A) 0 h



B) 24 h, 40 ˚C



C) 24 h, 70˚C

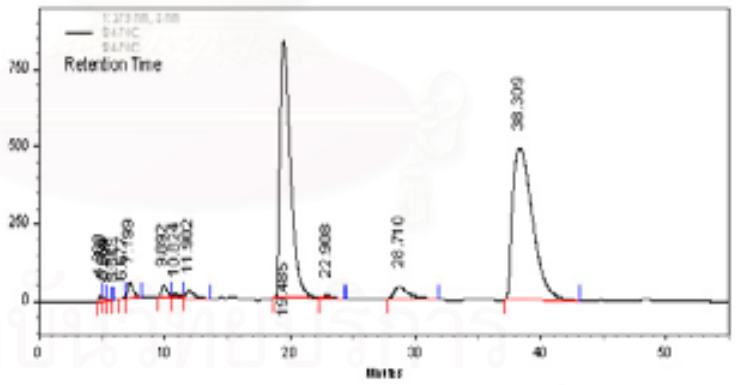


Figure 18 HPLC chromatogram of reaction products of CGTase (80 U/ml) with 0.5% EC and 1.0% beta-CD at different temperatures

A) 0 h

B) 24 h, 40 ˚C

C) 24 h, 70 ˚C

Table 16 The amount and the ratio of catechin to epicatechin in reaction mixtures incubated at 40 °C and 70 °C

Condition	Catechin* (%)	Epicatechin* (%)	Catechin : Epicatechin
0 h	8.3	91.1	1 : 11
24 h, 40 °C	8.17	68.8	1 : 8.4
24 h, 70 °C	44.0	48.6	1 : 1.1

* The amount was determined from peak area of each compound as compared to total peak area

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3.3.6 Effect of incubation time

Using the conditions as mentioned in 3.3.5 with various incubation times, it was found that product yields were clearly affected. The percent yields of each transglucosylated product and total product yields were shown in Table 17 and Figure 19. The total product yield at 12 hours incubation was rather low when compared with 24 hours. The maximum total product yield was obtained at around 18.07-18.70% when incubation time was in the range of 24-60 hours. To save on time, the 24 hour was chosen for the optimum incubation time. When considering of the yield of each product, the maximum amounts of Rt 48 and 45 min were observed at 24-36 hours while those of Rt 30 and 27 min were at 36 hours. The relative yield was in the order of Rt 48 min >> Rt 45 min > Rt30 min >> Rt 27 min. The maximum product of Rt 28 min was at 60 hours of incubation.

The epimerization was also noticed when varying of incubation time. From HPLC profile, at time zero of incubation, the peak area of catechin was about 7.5% of total area (Figure 20A). After incubation at 50 °C for 24 hours, the amount of catechin was 8.7% (Figure 20B). While at 60 hours, the catechin was increased to 12% (Figure 20C). This suggested that the epimerization of epicatechin to catechin was also an effect of longer incubation time (Table 18). However, the effect from alkaline pH and high temperature was much more pronounced.

Therefore, the optimum condition for transglucosylation by CGTase from *Paenibacillus* sp. RB01 which gave total EC glucoside products yield at the maximum was: incubation of 80U/ml of CGTase with 0.1% beta-CD and 0.5% epicatechin in 10 mM acetate buffer, pH 6.0 at 50 ºC for 24 hours. The 18.07% of total transglucosylation yield consisted of Rt27 1.44%, Rt30 3.39%, Rt45 4.18% and Rt48 8.33%, respectively. The profiles of reaction products before and after optimization were compared in Figure 21. Though the condition chosen was not closed to optimum for Rt 28 min, which required higher temperature and longer incubation time, its yield was 1.03%. Only the major products, Rt45 and Rt48, were further characterized and determined for their properties.

Table 17 Percent yields of transglucosylated products at different incubation time

Incubation time (hour)	Product yield (%)					Total yield (%)
	Rt 27	Rt 28	Rt 30	Rt 45	Rt 48	
12	0.80	-	3.16	3.31	5.84	13.11
24	1.44	0.73	3.39	4.18	8.33	18.07
36	1.70	1.03	3.71	4.21	8.26	18.70
48	1.35	1.35	3.70	3.98	7.76	18.14
60	1.38	1.90	3.72	3.62	7.88	18.51

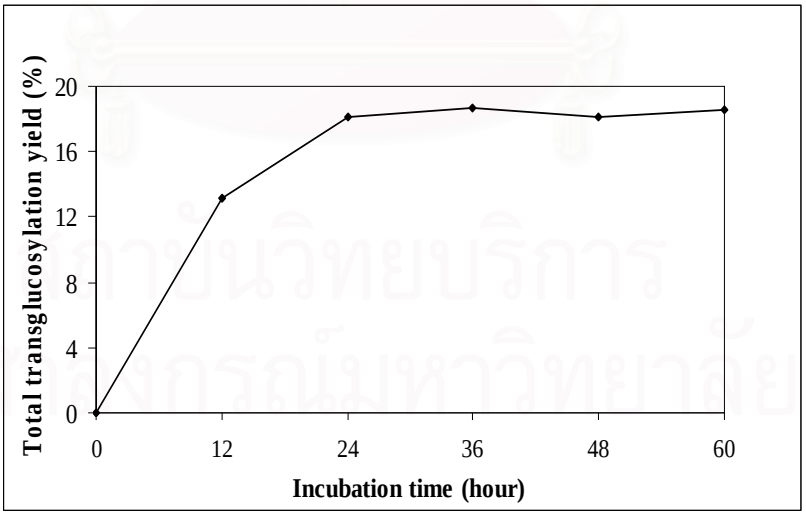
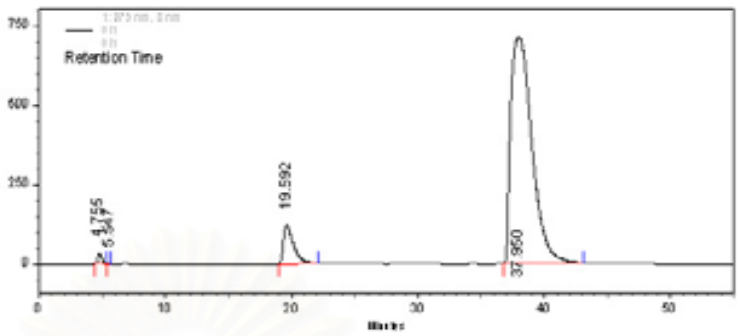
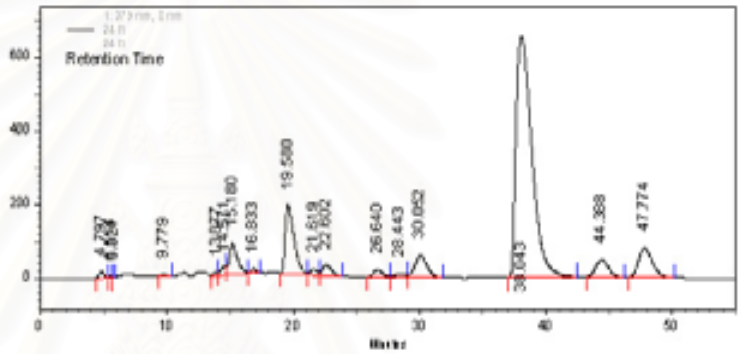


Figure 19 Effect of incubation time on transglucosylation yield

A) 0 h



B) 24 h



C) 60 h

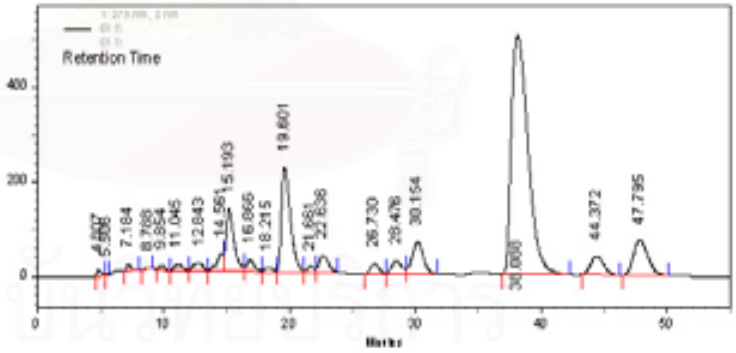


Figure 20 HPLC chromatogram of reaction products of CGTase (80 U/ml) with 0.5% EC and 1.0% beta-CD at 50 ˚C

A) 0 h

B) 24 h

C) 60 h

Table 18 The amount and the ratio of catechin to epicatechin in reaction mixtures at 50 °C for different incubation times

Incubation time	Catechin* (%)	Epicatechin* (%)	Catechin : Epicatechin
0 h	7.5	91.7	1 : 12.4
24 h	8.7	65.4	1 : 7.5
60 h	12.5	53.9	1 : 4.3

* The amount was determined from peak area of each compound as compared to total peak area

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A)

B)

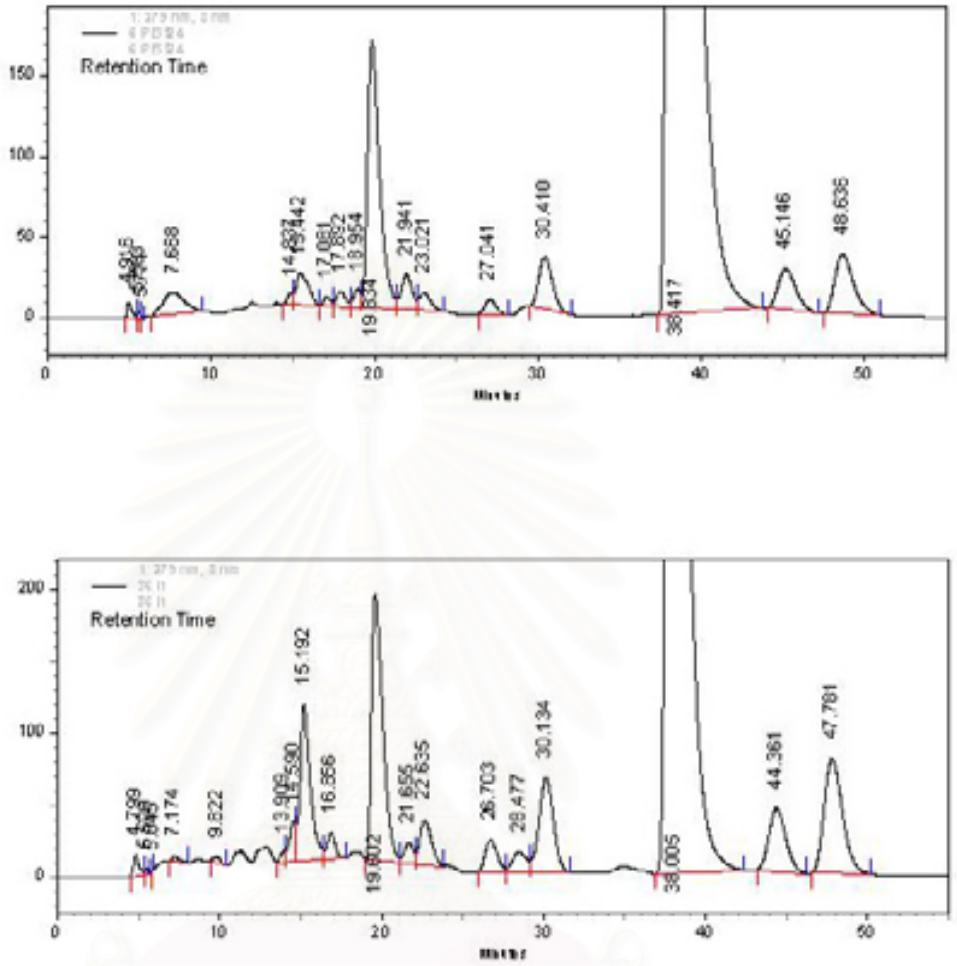


Figure 21 HPLC chromatogram of reaction products of CGTase with EC and beta-CD

A) before optimization

B) after optimization

3.4 Larger scale preparation and isolation of glucosylated products

The increased scale of reaction mixture (50 ml) was prepared as described in 2.13 using optimum condition for transglucosylation obtained in section 3.3. After transglucosylation reaction, the reaction mixture was concentrated to 5 ml with a rotary evaporator at 45 $^{\circ}$C and then applied on Sephadex LH-20 column. The column was firstly eluted with 2 litres of distilled water and then methanol, at a flow rate of 30 ml/h. The collected fractions were measured spectrophotometrically for EC at a wavelength of 279 nm while the fractions containing sugars were detected by phenol-sulphuric method and read absorbance at 480 nm. The Sephadex LH-20 column profile of reaction products was shown in Figure 22. When the column was eluted with distilled water, sugars and enzyme were firstly removed from the reaction products (those high peaks during fraction number 40-100) and there were 5 main separate peaks (peak I to V, during fraction number 300-820) came after them. After that the column was eluted by 50% methanol and the two peaks with high A_{279} (peak VI and VII) were observed. Each peak was analyzed by HPLC as described in the section 2.10.3.

From HPLC chromatogram (Figure 23), peak I to V were composed of more than one product while peak VI and VII were epicatechin and catechin, respectively (Table 19). Peaks III and IV consisted of Rt 45 and Rt 48 min products. From Sephadex LH-20 profile (Figure 22), peak III and IV were major peaks among EC derivatives eluted by water (peak I-V). And from HPLC profile of peak III and IV (Figure 23C and 23D), the peaks at Rt 45 and 48 min constituted for 35% and 65% of total products in those peaks. Therefore, peaks III and IV were pooled and concentrated with a rotary evaporator. The concentrates were then purified again by HPLC using C18 reversed phase column and glucoside products, the Rt 48 and 45 min, were collected by HPLC fraction collector. The glucoside products were further characterized for sizes and structures and determined for their properties.

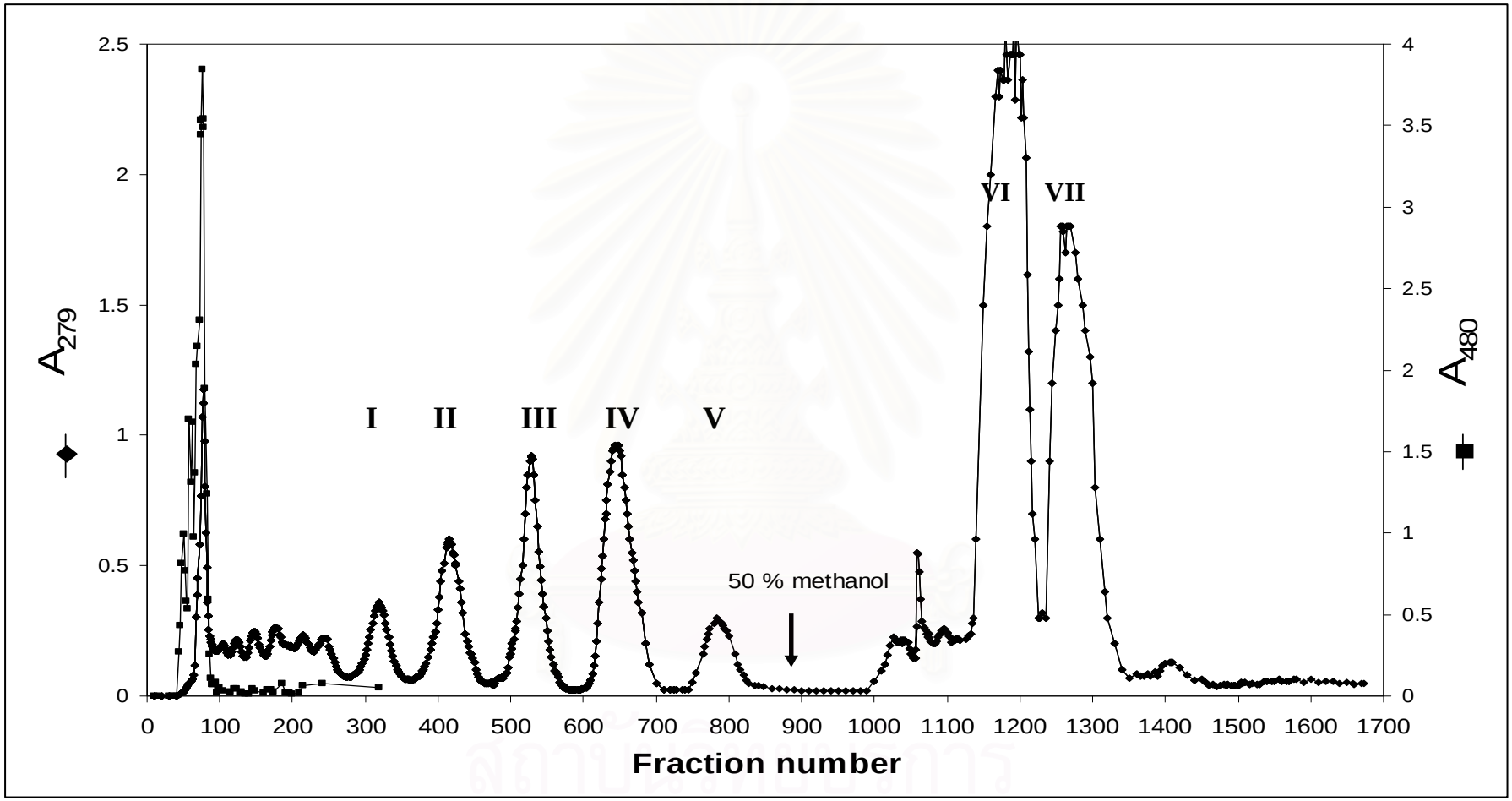
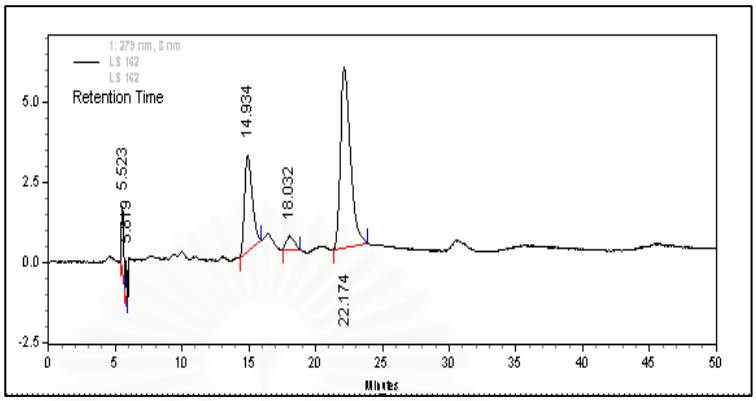
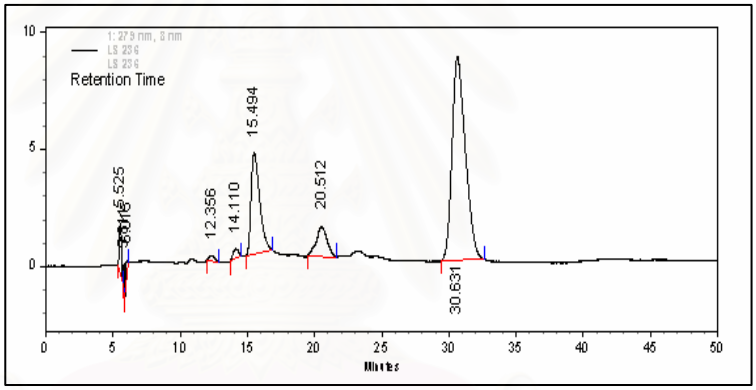


Figure 22 Sephadex LH-20 column profile of EC reaction products, column size 55x2.5 cm, flow rate 30 ml/h, fraction size 2 ml, water and 50% methanol as eluent

A) peak I



B) peak II



C) peak III

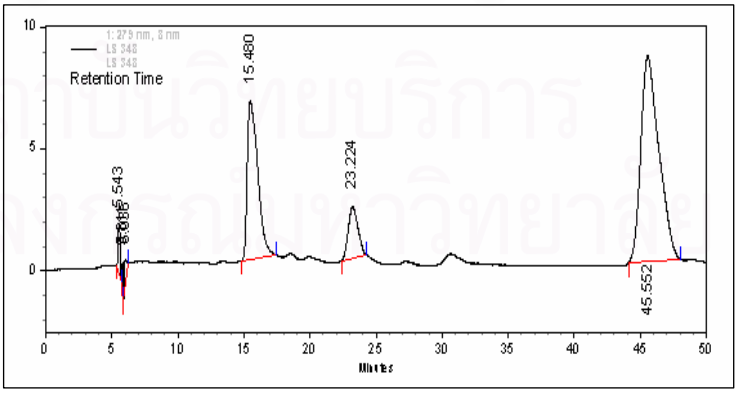
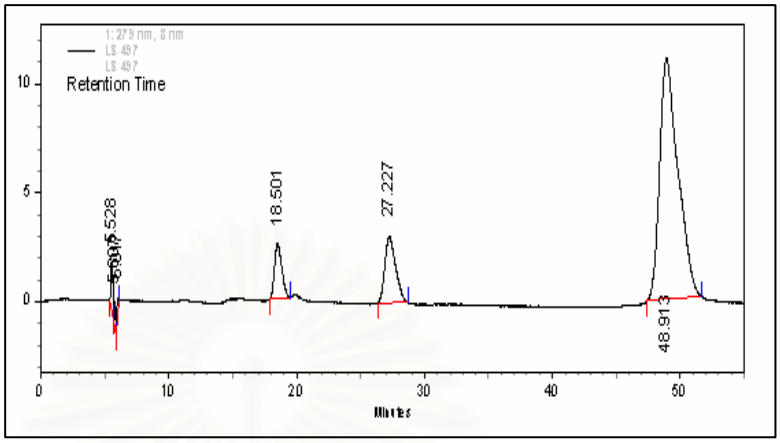
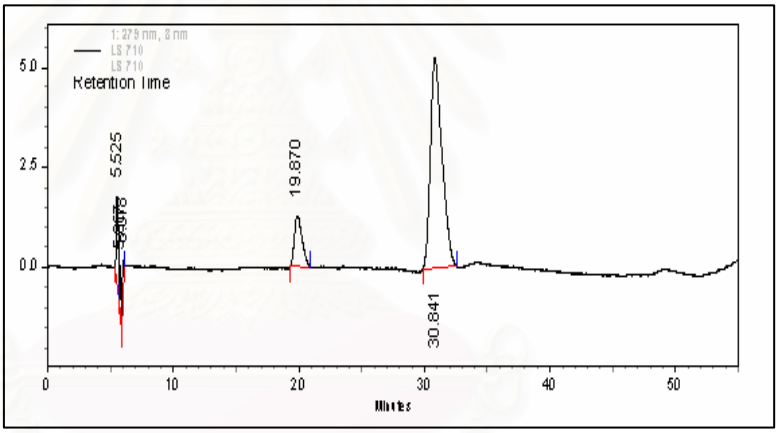


Figure 23 HPLC chromatogram of peaks I to VII collected from Sephadex LH-20 column

D) peak IV



E) peak V



F) peak VI

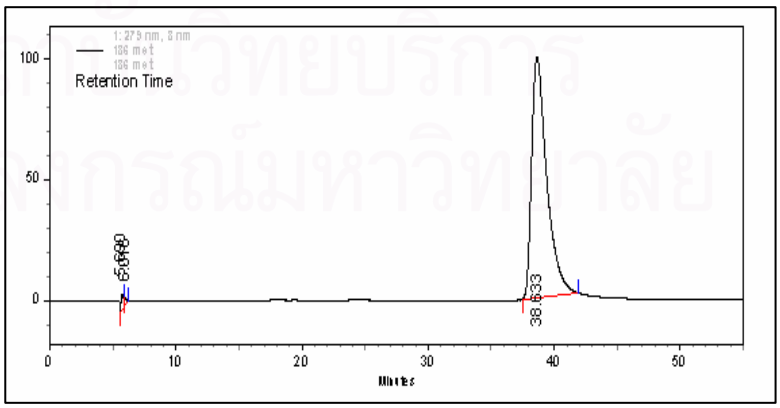


Figure 23 HPLC chromatogram of peaks I to VII collected from Sephadex LH- 20 column (Continued)

G) peak VII

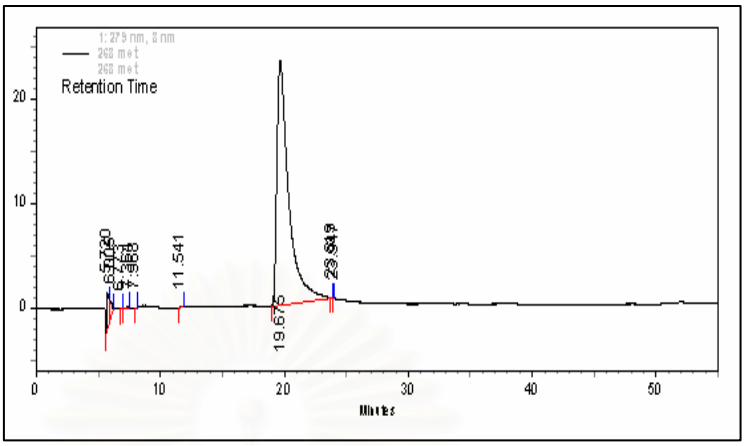


Figure 23 HPLC chromatogram of peaks I to VII separated from Sephadex LH- 20 column (Continued)

Table 19 Identification of peaks I to VII collected from Sephadex LH-20

Peak	Rt
I	15, 18, 22
II	15, 20**, 30*
III	15, 23, 45*
IV	18, 27*, 48*
V	20**, 30*
VI	38***
VII	20**

* Rt27, 30, 45, 48 - major EC glucoside products (as proved in Figure 10-B and Figure 11)

** Rt20 - catechin

*** Rt38 - epicatechin

3.5 Characterization of EC glucosides

3.5.1 Mass Spectrometry

The molecular weights of synthesized products were elucidated by mass spectrometer as described in section 2.14.1. The molecular weight of the product at Rt 48 min was estimated to be 452 daltons by ESI-TOF mass spectrometry with $[M+Na]^+$ at *m/z* of 475. This corresponded to the size of epicatechin monoglucoside. However, this product also exhibited the ion peak at *m/z* of 927 which corresponded to the dimer form of the product (904 daltons) (Figure 24). The spectrum of standard EC (Figure 26) with the molecular weight 290 showed the signal at *m/z* of 313, with the higher signal at *m/z* of 603 (a dimer form), and a lower signal at m/z of 893 (a trimer form). For the product at Rt 45 min, the molecular weight was estimated to be 614 dalton with $[M+Na]^+$ at *m/z* of 637 (Figure 25) and this corresponded to the size of epicatechin diglucoside.

3.5.2 Nuclear Magnetic Resonance

In ^{1}H-NMR spectrum, proton signals for glucoside products (the product at Rt 48 and 45 min) were assigned to the epicatechin, glucose and maltose in comparison with those of standard epicatechin, D-glucose and D-maltose (Table 20). The ^{1}H-NMR spectrum of the Rt 48 min (Figure 27) showed peak in the range of 3.40-3.80 ppm, characteristic of sugar protons. While a doublet signal at 7.30 ppm and a doublet-doublet signal at 6.97 ppm were assigned to the H-2′ and H-6′ of the epicatechin moiety, respectively. Due to the glycosylation of epicatechin, these proton signals showed downfield shifts by 0.42 and 0.29 ppm, respectively in comparison with standard epicatechin (Figure 29). It indicated the presence of a glycosidic bond on the 3′-hydroxy group at pyrogallol ring of the epicatechin moiety (Kiato *et al.*, 1993). The signal for the anomeric proton of sugar moiety appeared at 5.28 ppm with a coupling constant (*J*) of 3.63 Hz. This signal had a smaller coupling constant (*J*=3.63 Hz) than that for β-glucosidic (*J*=6-9 Hz). Therefore, this glucosyl residue was connected to epicatechin by α-linkage. Observation of the similarity of the ^{1}H-NMR for H-4, H-6 and H-8 position with epicatechin, suggested that substitution of

the glucose molecule did not take place at the 3, 5 or 7 position of epicatechin. Consequently, the product at Rt 48 min was identified as (-) epicatechin-3′-*O*-α-D-glucopyranoside (Figure 30).

Proton signals of the product at Rt 45 min (Figure 28) were similar to spectrum of the Rt 48 min. A doublet signal at 7.42 ppm and a doublet-doublet signal at 7.06 ppm were assigned to the H-2′ and H-6′ of the epicatechin moiety and showed downfield shifts by 0.54 and 0.38 ppm, respectively. The signal for the anomeric proton of sugar moiety appeared at 5.35 ppm with a coupling constant (*J*) of 3.58 Hz. The substitution of the glucose molecule did not take place at the 3, 5 or 7 position of epicatechin due to the similarity of the ^{1}H-NMR for H-4, H-6 and H-8 position with epicatechin. Therefore, this diglucosyl residue was connected to epicatechin by α-linkage and attached at the same position on epicatechin with the glucosyl residue of the Rt 48 min. However, the signal protons for maltose were similar to glucose. Therefore, the maltose molecule of the Rt 45 min could not be distinguished from the glucose molecule of the Rt 48 min by ^{1}H-NMR. But from the MS result in section 3.5.1, it confirmed that the Rt 45 min was a diglucoside. Consequently, the product at Rt 45 min was identified as (-) epicatechin-3′-*O*-α-D-diglucopyranoside (Figure 31).

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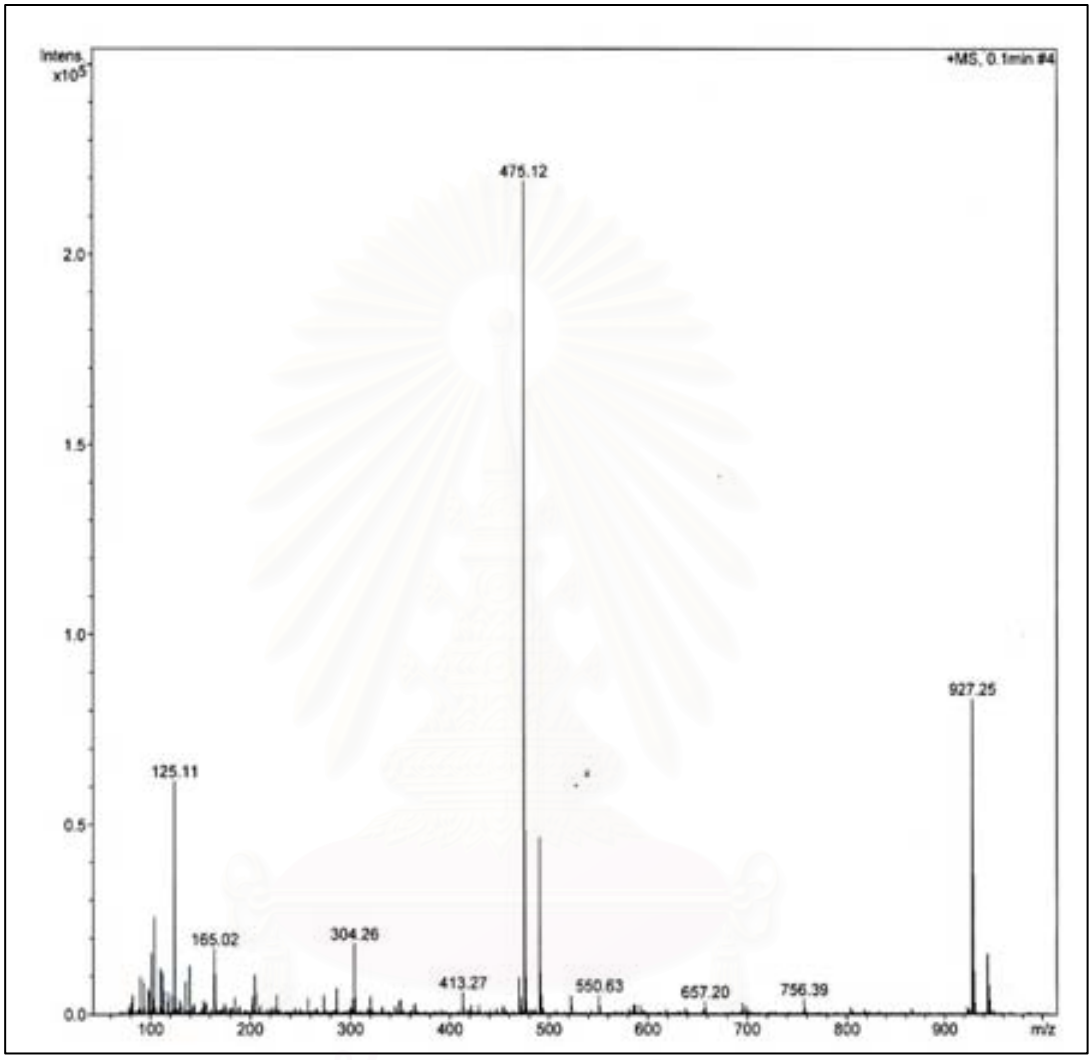

Figure 24 ESI-TOF mass spectrum of the product at Rt 48 min

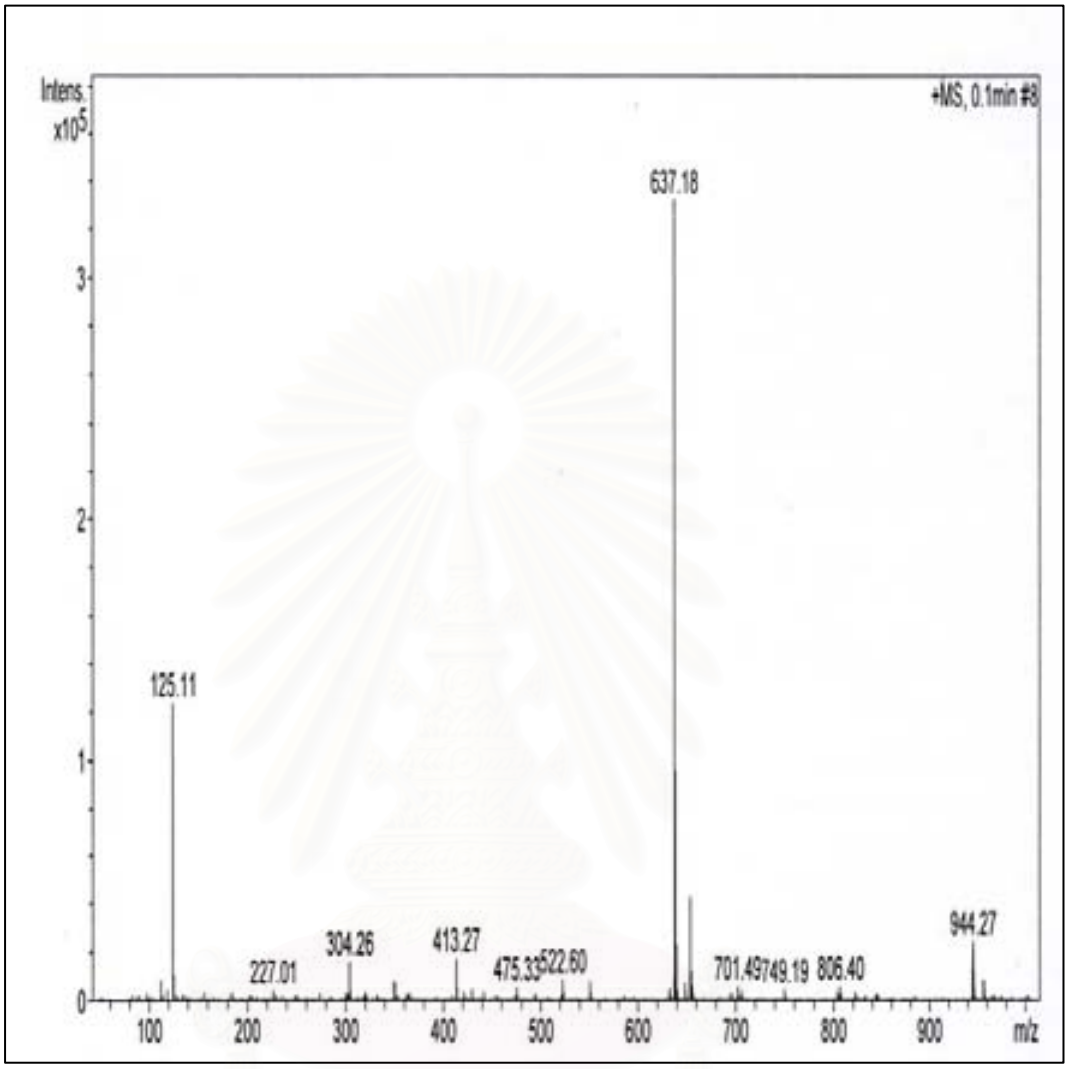

Figure 25 ESI-TOF mass spectrum of the product at Rt 45 min

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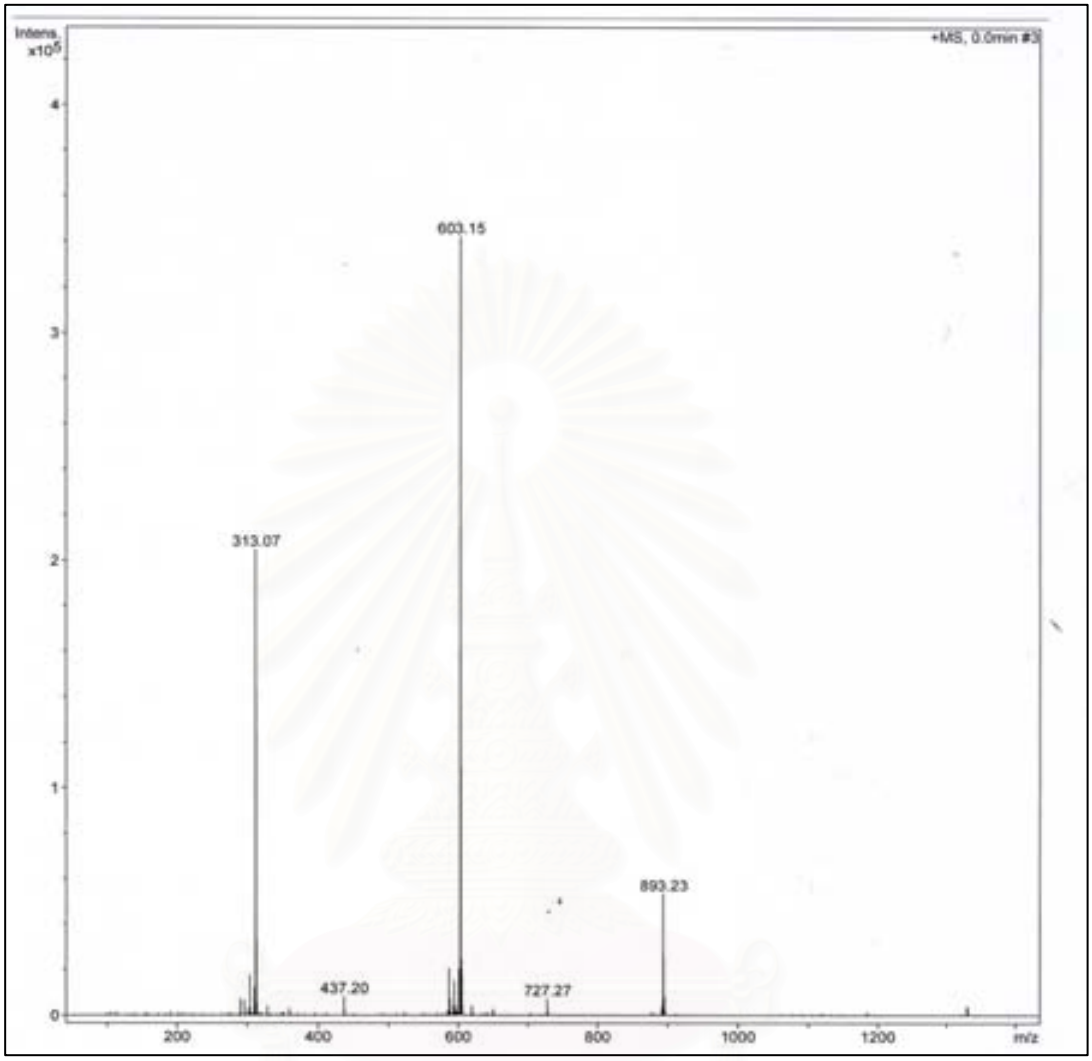

Figure 26 ESI-TOF mass spectrum of standard epicatechin

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Table 20 ^{1}H-NMR data of epicatechin glucosides (the products at Rt 48 and Rt 45 min)

Position	δ_H	
	Rt 48 min	Rt 45 min
Epicatechin		
2	4.51	4.59
3	4.10	4.19
4	2.61-2.80	2.71-2.89
5	-	-
6	5.82	5.92
7	-	-
8	5.85	5.94
9	-	-
10	-	-
1’	-	-
2’	7.30	7.42
3’	-	-
4’	-	-
5’	6.76	6.85
6’	6.97	7.06
Glucose (maltose)		
1”	5.28 (J = 3.63 Hz)	5.29 (J = 3.62 Hz)
2”	3.39	3.57
3”	3.58	3.89
4”	3.24	3.59
5”	3.70	3.72
6”	3.75	3.76

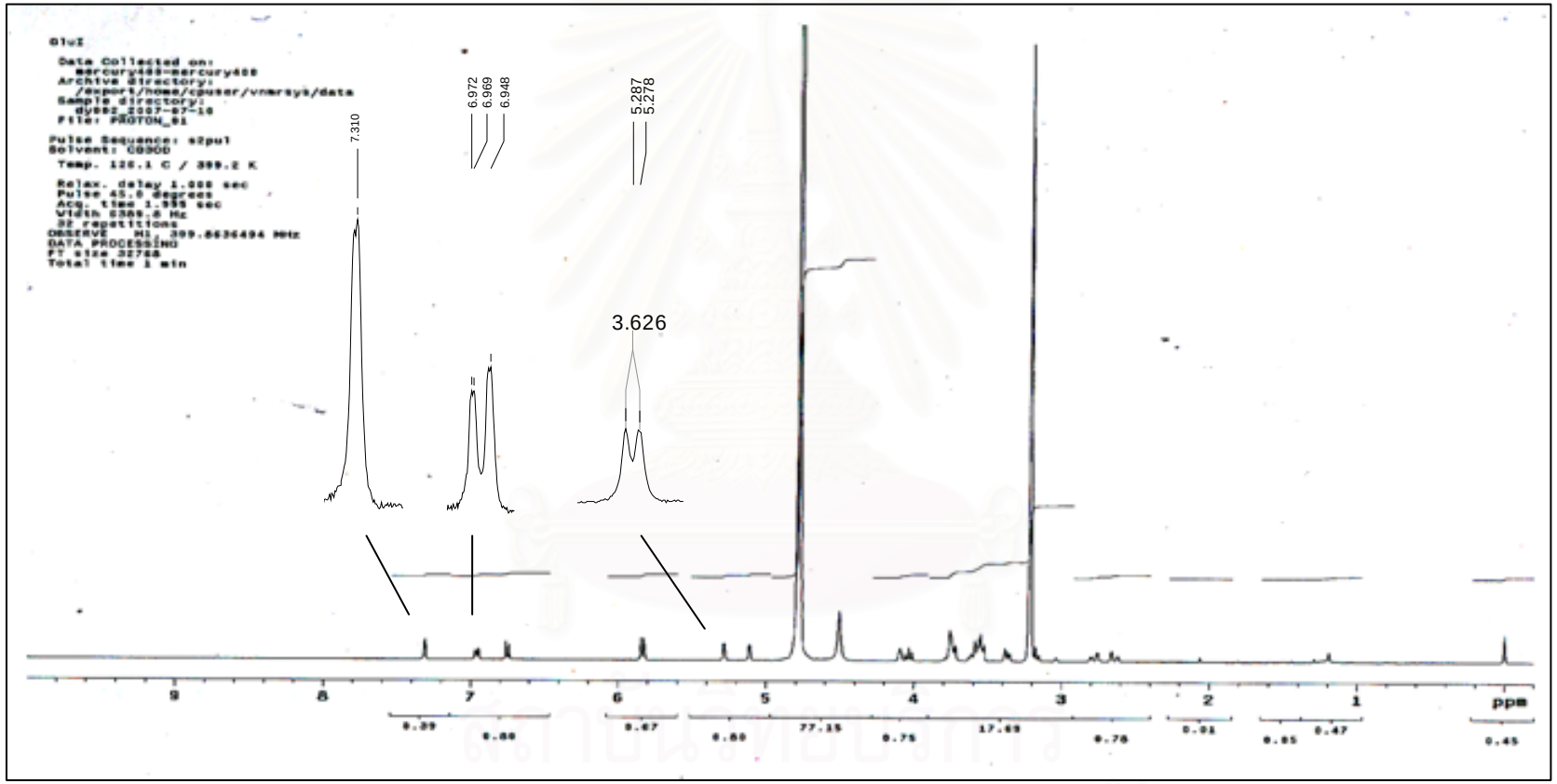


Figure 27 The 400 MHz ^{1}H-NMR spectrum of the product at Rt 48 min

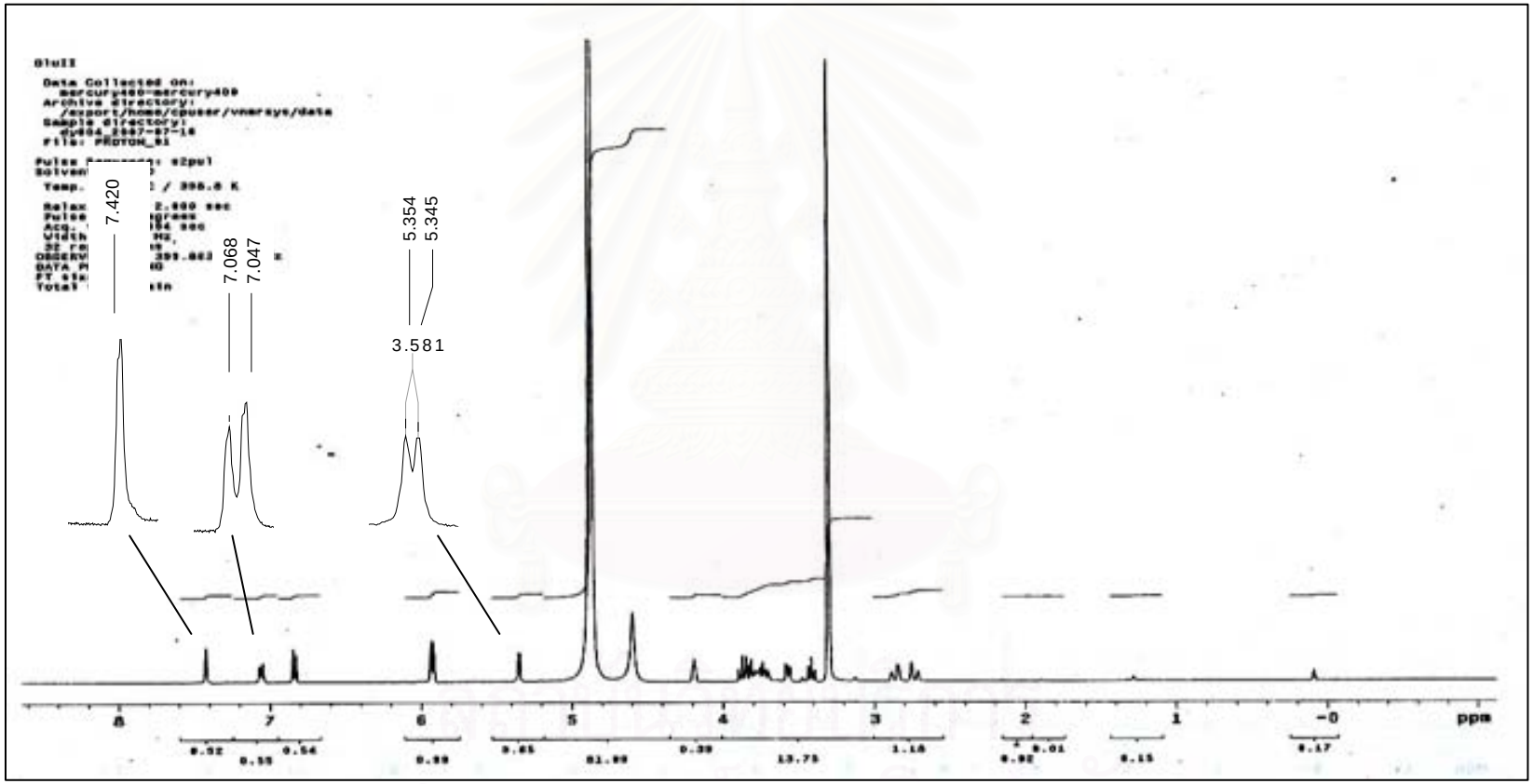


Figure 28 The 400 MHz ^{1}H-NMR spectrum of the product at Rt 45 min

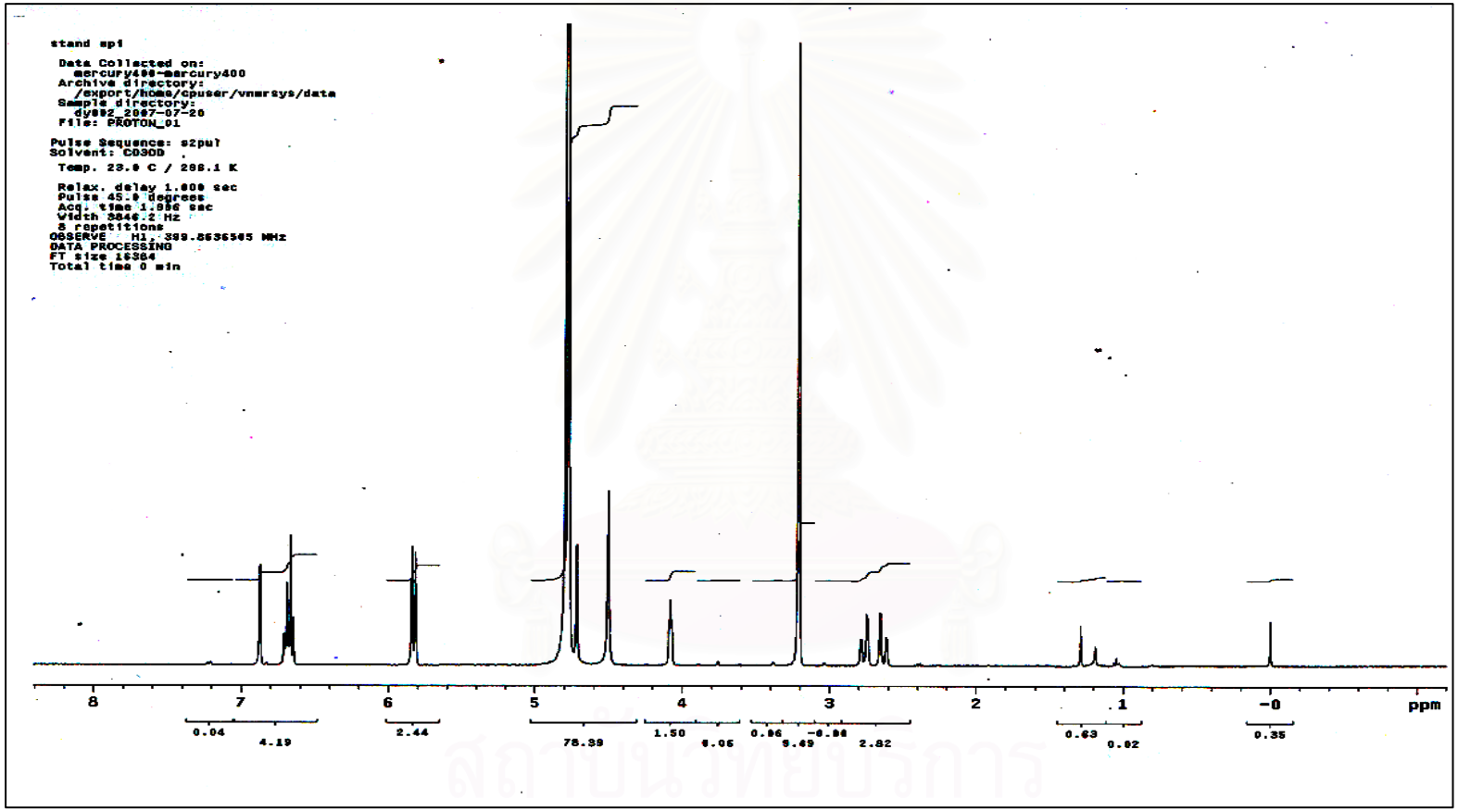


Figure 29 The 400 MHz ^{1}H-NMR spectrum of epicatechin

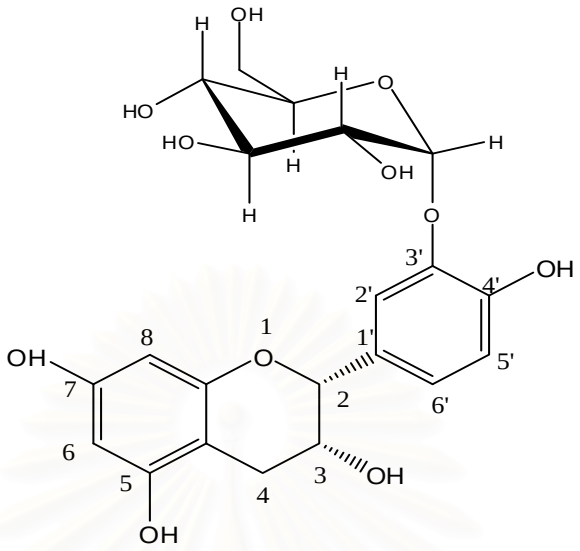


Figure 30 Proposed structure of the product at Rt 48 min, (-) epicatechin-3′-*O*-α-D-glucopyranoside

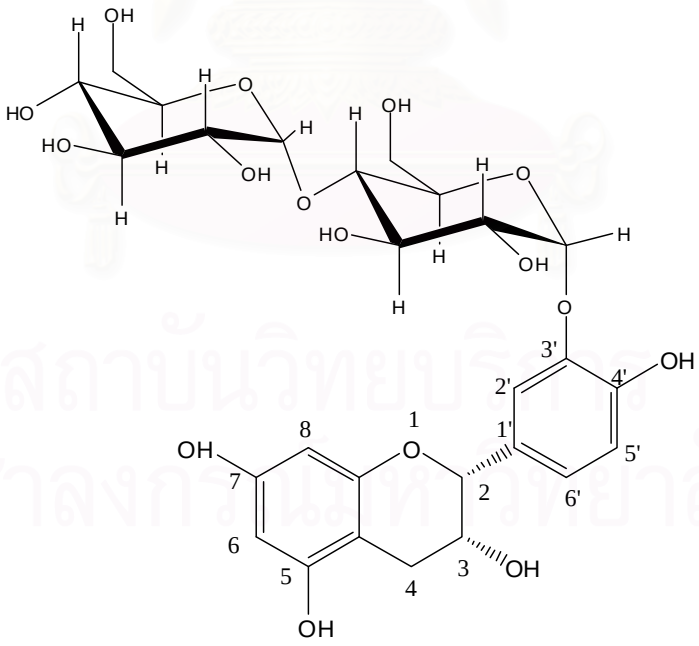


Figure 31 Proposed structure of the product at Rt 45 min, (-) epicatechin-3′-*O*-α-D-diglucopyranoside

3.6 Determination of properties of EC glucosides

3.6.1 Solubility in water

The solubility of each epicatechin glucoside was compared to that of epicatechin. The excess EC and EC glucosides were mixed with water and incubated at 30 °C for 15 minutes. The soluble part of samples was analyzed by HPLC for determination of the concentrations. From the result, the solubility of epicatechin was 4.9 mg/ml whereas the solubility of epicatechin monoglucoside and diglucoside were 95 and 2 mg/ml, corresponding to 19 and 0.4 times higher than that of epicatechin.

3.6.2 Browning resistance

The 0.1% of epicatechin, epicatechin monoglucoside and diglucoside in water solution were exposed to UV irradiation for 10 hours. The absorbance at 460 nm was measured every 2 hours and the increase in absorbance was plotted against time (Figure 32). Epicatechin, epicatechin monoglucoside and diglucoside showed a rise in absorbance at the first 2 hours of irradiation. After that epicatechin absorbance remained constant and rapidly increased after 6 hours of irradiation while the absorbance of epicatechin diglucoside gradually increased and stayed at the level twice the value at 2 hours. By contrast, after 2 hours of irradiation, the absorbance of epicatechin monoglucoside was hardly increased.

3.6.3 Antioxidant activity

The antioxidant activity of EC, epicatechin monoglucoside and diglucoside were evaluated using DPPH radical scavenging assay. Each of the samples dissolved in ethanol at various concentrations was mixed thoroughly with 100 μM DPPH. The absorbance of the mixture was measured at 517 nm after 10 minutes incubation in the dark. DPPH radical scavenging activities of each sample were expressed as the percentage of DPPH decrease in the absorbance compared to a blank (Figure 33). The IC_{50} value designates the inhibitory concentration at which the absorbance was reduced by 50%. The IC_{50} of epicatechin, epicatechin monoglucoside and diglucoside were 25, 36 and 46 μM, respectively.

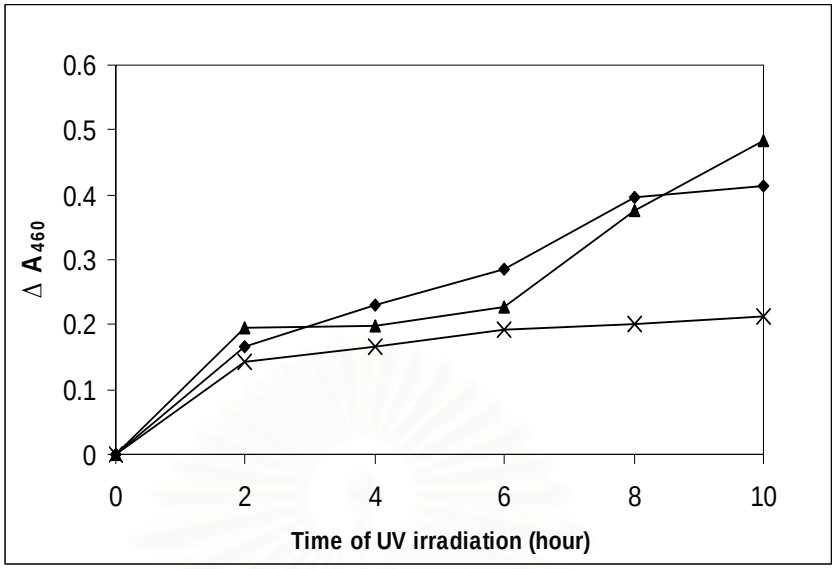


Figure 32 Browning resistance to UV irradiation of epicatechin and epicatechin glucosides A solution containing 0.1% (w/v) EC (▲), EC monoglucoside (×), or EC diglucoside (◆) was exposed to UV irradiation and the absorbance at 460 nm was continuously monitored.

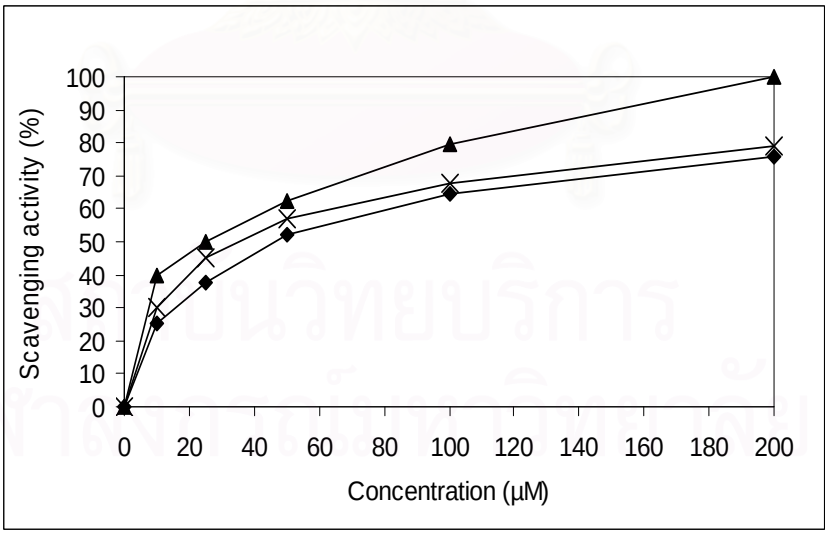


Figure 33 DPPH radical-scavenging activities of epicatechin and epicatechin glucosides EC (▲), EC monoglucoside (×), or EC diglucoside (◆) at various concentrations was mixed with DPPH and then the absorbance was monitored at 517 nm.

CHAPTER IV

DISCUSSION

4.1 Purification of CGTase

Paenibacillus sp. RB01, the thermotolerant bacteria producing CGTase, was screened from hot spring area in Ratchaburi province, Thailand. The optimum condition for growth and for producing CGTase of these bacteria was previously performed and reported by Tesana (2001). Since CGTase are extracellular enzyme, the enzyme was secreted out of the cells into the medium and the crude CGTase was partially purified by starch adsorption method (Kato and Horikoshi, 1985, modified by Laloknam, 1997). This method based on the interaction between substrate and enzyme or substrate-enzyme affinity binding. CGTase was adsorbed by corn starch added into the crude enzyme and the adsorbed enzyme was then eluted with maltose in buffer solution. Maltose, one of the substrates of CGTase, has higher affinity to CGTase than starch. Therefore, maltose was added into the starch adsorbed enzyme for competitive binding of CGTase. Consequently, enzyme-substrate that presented in soluble fraction was easily separated from pellet of starch. The enzyme-maltose solution was dialyzed against buffer solution to get rid of maltose from enzyme and the enzyme activity was determined by dextrinizing assay. Since CGTase will degrade starch into oligasaccharides which cannot form complex with iodine, the enzyme activity is defined as amount of enzyme producing the reduction in the intensity of starch-iodine complex. From partial purification table (Table 7), the enzyme with 69.8% yield and 14.6 fold increase in purity was obtained. While the previous work on the starch adsorbed CGTase from *Paenibacillus* sp. RB01 by Yenpetch (2000) and Tesana (2001) gave 72% and 57.3% yield with 29 and 26.8 fold increases in purity. From SDS-PAGE (Figure 6), the CGTase band (65 kDa, Yenpetch, 2000) was approximately 2/3 of total proteins and only one protein band of lower molecular weight was contaminated. Thus, the purity of partially purified enzyme was high enough for using in further transglucosylation study.

4.2 Synthesis of catechins glucosides and detection of the products

After incubation of CGTase with catechins acceptors and beta-CD donor, the expected glucosylated products were detected by TLC. This chromatography was used to basically detect the synthesized glucoside products. TLC plate is composed of silica gel as a polar stationary phase, while catechins, non-polar compounds, are weakly adsorbed with. Catechins, therefore, were migrated to the solvent front (the larger Rf) with the non-polar mobile phase (ethyl acetate: acetic acid: water, 3:1:1) and the oligosaccharides, the polar compounds, are strongly adsorbed with siliga gel. They slightly moved from the origin (the smaller Rf). And the expected catechins glucosides, more polar than catechins but less polar than oligosaccharides, were migrated between these two compounds. While in the control condition (no catechins, only CGTase and beta-CD were present), glucosides were not detected. The visualization of spot on TLC plate then was done by charring reagent. This step involves spraying TLC plate with sulfuric acid in methanol, followed by heating the layer at relatively high temperature to degrade organic compounds into black or brown zones of carbon on a white background. Thus, the charring reaction is non-specific and sometimes is called universal detection (Wall, 2005).

Each catechin acceptor gave various number of glucoside products (Figure 7). On TLC plate, catechin and epigallocatechin gallate gave at least three products while epicatechin gave at least four products, the two minor and the two major. In the previous work of Funayama *et al.* (1993), at least four products of catechin glucoside were detected by TLC. These glucosides were synthesized from *Bacillus macerans* CGTase which was an α-CGTase while our *Paenibacillus* sp. CGTase was a β-CGTase. This suggested that different types of CGTase might result in different number of products. The other enzymes used in transglucosylation of catechins had also been reported. Kitao *et al.* (1993) synthesized glucosides of various catechins by transglycosylation with *Leuconostoc mesenteroids* sucrose phosphorylase. Only one catechin glucoside was detected while in case of epicatechin and epigallocatechin gallate acceptors, two or more products were detected. Moon *et al*. (2006) detected three EGCg glucosides by transglucosylation with glucansucrase from *Leuconostoc mesenteroid*.

When compared acceptor specificity of the three catechins (C, EC and EGCg) by TLC (Figure 7 and Table 9), the spot intensity of the products using EGCg as an acceptor was stronger than of EC and C, respectively. Therefore, EGCg shows higher acceptor specificity to CGTase. This suggested that *Paenibacillus* sp. RB01 CGTase acts differently in catalyzing transglucosylation reaction in comparison with *Bacillus subtilis* X-23 α-amylase (hydroquinone glucosylating enzyme) which demonstrated similar extent of acceptor specificity among different catechins (Nishimura *et al.*, 1994). Though EGCg contains higher hydroxyl groups than C or EC, this does not take into account for its higher specificity as acceptor, as evidenced by similar work of Funayama *et al.* (1994). They demonstrated that an acceptor with one hydroxyl group (e.g. *p*-methoxy phenol) can be as efficient as acceptor with five hydroxyls (e.g. catechin) in the formation of glucosides catalyzed by glucoside synthetase from *Bacillus subtilis* K-531. In addition, the acceptor specificity of sucrose phosphorylase from *Leuconostoc mesenteriods* (Kitao *et al*., 1993) decreased in the following order: ECg ≈ C > EC > EGCg > EGC.

When starch, beta-CD, and G7 were used as donor and EC was used as acceptor in transglucosylation reaction with CGTase. TLC chromatogram shows the same degree of spot intensity of products in all cases (Figure 8). While transglucosylation efficiencies when using the three donors as determined by measuring disappearance of acceptor using HPLC and expressed as percent transglucosylation yield were not significantly different. The two methods thus confirm that these substrates were equally effective as glucosyl donor for this CGTase.

From TLC chromatogram (Figure 8), the two major spots of epicatechin glucosides (named Glu I and Glu II) were detected. While the reaction products analyzed by HPLC showed the peaks of products at Rt 27, 30, 45, and 48 min. These peaks of products collected from HPLC fraction collector were compared with Glu I and II in TLC. From TLC chromatogram (Figure 11), the spots of products at Rt 48 and 45 min had Rf equal to Glu I and Glu II whereas the spots of products at Rt 30 and 27 min had Rf slightly lower than Glu I and Glu II, respectively. Thus Glu I was the mixture products of Rt 30 and 48 min (Rt 48 > Rt 30) and Glu II also was the

mixture products of Rt 27 and 45 min (Rt 45 > Rt 27) as evidenced by HPLC profiles (Appendix 5).

The number of glucosyl units and the configuration of the products were basically characterized by the action of glucoamylase and α-glucosidase, respectively. Since glucoamylase (amyloglucosidase) (E.C. 3.2.1.3) is an inverting, exohydrolase enzyme which hydrolyzes the terminal 1, 4-linked alpha-D-glucose residues from non-reducing ends of the chains with the release of β-D-glucose (Meager *et al.*, 1989), the reaction products expected to be oligoglucosides were hydrolyzed to monoglucosides and glucose molecules. From TLC result (Figure 9, lane d), after treated with glucoamylase, Glu II was disappeared while the increased intensities of Glu I and glucose spots were observed. Similar to analysis by HPLC (Figure 10C), the products at Rt 27 and 45 min were disappeared after treated with glucoamylase while products at Rt 30 and 48 min were increased. Therefore, Glu II, Rt 27 and 45 min were suggested as EC oligoglucosides. While α-glucosidase (E.C. 3.2.1.20), a retaining exohydrolase, catalyzes the hydrolysis of terminal, non-reducing 1, 4-linked α-D-glucose residues with the release of alpha-D-glucose. The glucoside products were thus further hydrolyzed by α-glucosidase in order to confirm linkage between EC and glucosyl unit. From TLC chromatogram (Figure 9, lane e), after treated with α-glucosidase, no glucoside spot was found while EC and glucose were recovered. Corresponding to the results from HPLC, the products at Rt 30 and 48 min were disappeared after treatment with α-glucosidase. This confirmed that Glu I, Rt 30 and 48 min were EC monoglucosides with α-configuration.

4.3 Optimization of transglucosylation reaction

Before the optimization of transglucosylation reaction, the total transglucosylation yield of epicatechin glucosides was 7.65% (Table 10). The yield of 18.07% was obtained after optimization (Table 17). The total transglucosylation yield of products thus was increased more than 2 folds after optimization. While the yield of Rt 48 min product, the major monoglucoside, was increased from 3.13 to 8.33%. The total yield of epicatechin glucosides using *Paenibacillus* sp. RB01 CGTase was approximately the same as total yield of catechin glucosides obtained by using

Bacillus macerans CGTase (Funayama *et al*., 1994). They reported that the 18.3% of total yield of catechin glucosides was obtained; however, the major monoglucoside product was only 4.3%.

The 1.0% (8.8 mM) of beta-CD and 0.5% (17.2 mM) of epicatechin were the optimum concentrations of donor and acceptor, so the mole ratio of beta-CD to epicatechin was 1:2. It was noticed that the products at Rt 48 and Rt 30 min were about 2 folds more than that of Rt 45 and Rt 27 min, respectively (Table 17). The ratio of monoglucoside (Rt 48 and Rt 30 min) to oligosaccharide products (Rt 45 and Rt 27 min) was about 2:1. This suggested that CGTase from *Paenibacillus* sp. RB01 favored monoglucoside synthesis rather than oligoglucoside. However, this also may be the result of hydrolytic activity of CGTase. After opening the CD ring, the resulting glucose or linear oligosaccharides was transferred to epicatechin acceptor in the enzyme reaction mixture. The oligoglucoside could be transferred to water in case of hydrolysis reaction. Therefore, besides the coupling reaction, the hydrolytic activity of CGTase also might influence the conversion of oligoglucoside to monoglucoside. However, hydrolytic activity of CGTase is considered to be rather low (Van der veen, 2000).

During optimization of transglucosylation reaction, the epimerization of epicatechin to catechin was observed at alkaline pH, high temperature and longer incubation time. Epimerization is a reaction commonly occurred between *trans*- and *cis*- isomers of a compound. The amount of epicatechin epimerized to catechin when the reaction mixture was incubated at high temperature was higher compared to the effect of alkaline pH and longer incubation time. This suggested that high temperature had the most influence to epimerization reaction. Therefore, the lowest total transglucosylated yield of products incubated at 70 $^{\circ}$C (only 2.63%) (Table 15) was not only caused by denaturation of enzyme at high temperature and long incubation time but also by epimerization. The epimerization brings about the decrease of epicatechin substrate and consequently the less production of epicatechin glucosides. Therefore, precautions have to be made in selecting experimental conditions for transglucosylation of epicatechin. This finding was similar to the work by Xu *et al.* (2003), who reported that four epimers of epicatechins: catechin (C), catechin gallate

(Cg), gallocatechin (GC) and gallocatechin gallate (GCg) in canned and bottled green tea drinks were not originally present in green tea leaf but that they were produced by thermal-induced epimerization reaction.

The product at Rt28 min, which could not be observed in HPLC profiles before optimization, was increased to 0.73% after optimization (Table 17). The maximum yield was 1.90% (Table 17). It is noticed that the Rt28 min product was only be detected and increased in alkaline pH, high temperature and longer incubation time, the condition which was not closed to optimum of epicatechin glucosides (Rt 27, 30, 45, and 48 min) Thus, it is likely that the product at Rt28 min was a catechin glucoside product.

4.4 Larger scale preparation and isolation of glucosylated products

To prepare higher amounts of glucoside products for characterization, 50 ml reaction mixtures were performed. The glucosylated products were isolated from the mixture of reaction products using the Sephadex LH-20 column. Sephadex LH-20 is prepared by hydroxylpropylation of Sephadex G-25, a bead-formed dextran medium, and has been specifically developed for gel filtration of natural products, such as steroids, terpenoids, lipids and low molecular weight peptides, in organic solvents (GE Healthcare, 2006). Each glucoside product was separated due to their different properties in size and polarity. From Sephadex LH-20 column profile of epicatechin reaction products (Figure 22), the fractions eluted with water in the fraction range of 1-100 were saccharides and enzyme. Since saccharides and enzyme were polar compounds and high molecular weight, respectively, they were eluted in the void volume range. The fractions in the range of 100-300 were still the enzyme while those of 300-800 were composed of five main separate peaks with less polar compounds relative to saccharides. The non-polar compounds in the reaction mixtures were further eluted with 50% methanol and the two obtained peaks were continuously analyzed by HPLC. From HPLC profile (Figure 23F-G), these two peaks were epicatechin and catechin remained in the reaction mixtures.

When each peak in the fraction range of 500-800 was analyzed by HPLC, the product at Rt15 min was found in peak I, II and III (Figure 23A-C). While the product at Rt 22 min was also found in peak I (Figure 23A). The products at Rt15 and 22 min should be catechin glucosides because the positions of these products in HPLC chromatogram appeared around the catechin (Figure 10B) and obviously seen in the high temperature condition. The product at Rt 30 min was found in peak II and V about 65% and 78%, respectively (Figure 23B, 23E). This implied that the Rt30 min was the mixtures of two different glucoside products that could not be separated by C18-reversed phase HPLC column, but could be separated by Sephadex LH-20 column. The Rt 45 and 48 min were found as the major peak in peak III (35%) and IV (65%), respectively (Figure 23C-D). While the Rt27 min also was found as small peak in peak IV (13%) (Figure 23D). The yield of the product at Rt48 min in the larger scale preparation was 5.6% which was lower than the yield (8.33%) in the small scale reaction (1 ml).

4.5 Characterization of glucosides

Using MS and NMR, the two major glucoside products, Rt 45 and 48 min, were identified as epicatechin-3′-*O*-α-D-diglucopyranoside and epicatechin-3′-*O*-α-D-glucopyranoside, respectively (Figure 30-31). The epicatechin monoglucoside structure from this study, catalyzed by *Paenibacillus* sp. RB01 CGTase (a β-CGTase), had the same linkage and configuration as those previously reported catechin glucosides: catechin 3′-*O*-α-D-glucopyranoside synthesized by tranglucosylation of CGTase from *B. macerans*, an α-CGTase (Funayama *et al*., 1994), by sucrose phosphorylase from *Leuconostoc mesenteroids* (Kitao *et al*., 1993), and by transferase activity from *Xanthomonas campestris* WU-9701 (Sato *et al.*, 2000). While Nakahara *et al*. (1995) reported the different structures of glucoside products, catechin 4′-*O*-α-D-glucopyranoside, obtained by glucosylation of catechin using glucosyltransferase from *Streptococcus sobrinus*. Similarly, catechin 4′-*O*-α-D-glucopyranoside and catechin 4′, 7-*O*-α-di-D-glucopyranoside were synthesized by glucosyltransferase from *Streptococcus* sp. (Meulenbeld *et al,* 1999). The attached glucosyl molecule on ring “A” (C-7) of catechin glucoside was reported for the first time while previous work mostly reported the glucosyl molecule attached on ring “B”. Moon *et al.* (2007)

characterized the epigallocatechin gallate 4"-mono, 7, 4"-di-, and 4', 4"-di-*O*-α-D-glucopyranoside products obtained by transglucosylation with glucansucrase from *Leuconostoc mesenteroid.* From these reports, it was evidenced that the attached position of glucosyl molecule to catechins acceptor was dependent on types and sources of enzyme. However, the catechins glucoside products of only α-configuration were obtained from enzymatic transglucosylation reaction. The β-linkage was found in the natural glucosides, for example the catechin 7-*O*-β-D-glucopyranoside isolated from barley (*Hordeum vulgare* L.) and malt (Friedrich and Galensa, 2002).

4.6 Determination of properties of EC glucosides

4.6.1 Water solubility

Water solubility of epicatechin glucosides was performed and the amount solubilized was analyzed by HPLC. The concentration of each product was then determined from standard curve of epicatechin (Appendix 6). At 30 °C, the water solubility of Rt 48 (epicatechin-3′-*O*-α-D-glucopyranoside) and Rt45 (epicatechin-3′-*O*-α-D-diglucopyranoside) were 19 and 0.4 times higher than that of epicatechin. For monoglucosides of catechins, the increase in solubility with different degree was reported by others. The solubility of catechin 3'-*O*-α-D-glucopyranoside was 50-fold and 100-fold higher than that of catechin as reported by two research groups (Kitao *et al*., 1993; Sato *et al*., 2000). Moon *et al*. (2006) reported the solubility of 4" epigallocatechin gallate (EGCg) monoglucoside to be 69 times higher than that of EGCg. The discrepancy between these solubility properties may come from the difference in structure of catechin derivatives and also experimental methods used.

From our result, it is surprising that the solubility of Rt45 min, the diglucoside, was lower than that of Rt48 min, the monoglucoside, and epicatechin. Having more glucosyl units attached should result in more polar compound with the increase in solubility. As in other works e.g. solubility of 7, 4"-di-, and 4', 4"-diglucosides of EGCg were 126, and 122 times higher than EGCg (Moon *et al.*,

2006). Solubility of our product was rechecked by using standard curve of each compound, of which the absorbance was measured spectrophotometrically.

To determine the λ_{max} and $\varepsilon_{\lambda max}$ of each compound, standard epicatechin, the product at Rt 48, and 45 min were used to make standard curves by spectrophotometeric method (Appendix 7-9). Only with epicatechin standard, the standard curve by HPLC technique was also performed (Appendix 6). It was observed that the λ_{max} for epicatechin was at 279 nm, while those for epicatechin glucosides was at 274 nm. However, the absorbance of the glucosides at 274 and 279 nm were slightly different, the standard curve of all compounds was then performed at 279 nm. The $\varepsilon_{\lambda max}$ of standard epicatechin determined from HPLC and UV-spectrophotometer were pretty closed. However, the $\varepsilon_{\lambda max}$ of each glucoside was obviously different from that of epicatechin (Table 21). Consequently, the solubility values varied, depending on the $\varepsilon_{\lambda max}$. From HPLC profile of solubilized diglucoside product at Rt 45 min, epimerization to Rt18 min was also observed (Table 21). The two peaks were thus combined when calculating total amount of Rt45 min solubilized. However, it is surprising that the solubility of diglucoside product was lower than standard epicatechin. We propose that H-bonding between glycosyl and epicatechin moieties might occur which led to the strained structure.

Table 21 The $\varepsilon_{\lambda max}$ from standard curve of epicatechin, the mono- and di-glucosides

Compound	$\varepsilon_{\lambda max}$ by HPLC	$\varepsilon_{\lambda max}$ by spectrophotometer	Solubility	
			mg/ml*	mg/ml**
Epicatechin	5,706	5,500	4.9	4.9
EC monoglucoside (Rt 48)	-	9,564	95	52.8
EC diglucoside (Rt 45)	-	12,087	1.54	0.7
Rt45→Rt18	-	-	0.46	0.2

* determined from standard curve of epicatechin by HPLC (UV detector, 279 nm)

** determined from standard curve of each compound by UV spectrophotometry (at 279 nm)

4.6.2 Browning resistance

The browning reaction was activated by UV irradiation of epicatechin and glucoside products, and the increase in absorbance at 460 nm was followed (Kitao, 1993). From the result (Figure 32), the browning of epicatechin was obviously increased after 6 hours. This suggested that epicatechin is susceptible to degradation and browning by UV irradiation in water. However, the A_{460} of epicatechin monoglucoside still stayed at the same level even after 10 hours. While the browning of epicatechin diglucoside was continuously increased after 8 hours of UV irradiation, and stayed at the same level after that. This suggested that the epicatechin monoglucoside was more resistant to UV irradiation than epicatechin diglucoside and epicatechin, respectively. It was considered that the browning resistance of catechins glucoside to light irradiation was due to a steric hindrance by glycosylation of the pyrogallol ring, since the browning of catechins was caused by the polymerization between these rings (Hashimoto *et al*., 1988). Kitao *et al*. (1993) reported that catechin was easily degraded with rapid browning. On the contrary, the browning resistance of catechin monoglucoside in their work was much increased even after 7 hours. The browning resistance property of catechin glucoside was also reported by Sato *et al*. (2000) that the catechin solution turned brown after standing for 24 hours in contact with air at 20 °C, while the catechin monoglucoside solution showed no color change.

4.6.3 Antioxidant activity

The free-radical scavenging activity of epicatechin and epicatechin glucoside was determined by the use of a stable free-radical, DPPH. When DPPH reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The change in color from deep-violet to light-yellow was measured at 517 nm (Miliauskas et al., 2004). Catechins liberate the hydrogen radical from hydroxyl groups of 3′-and 4′-position in B ring and the hydrogen radical joins other radicals, by which this radical is stabilized (Koketsu, 1997). From scavenging activity assay (Figure 33), the inhibitory concentration at which the absorbance was reduced by 50% (IC_{50}) of epicatechin, epicatechin monoglucoside and diglucoside were 25, 36 and 48 μM, respectively. The

IC_{50} of epicatechin and catechin reported by Li *et al.* (2007) was 22 μM. The IC_{50} of mono and diglucoside from this study were 1.4 and 1.9 fold higher than that of epicatechin. This indicated that the antioxidant properties of products were dependent on their structure and the reduced scavenging activity was observed in the epicatechin glucosides.

These results were confirmed by Bar *et al.*'s work (1990). It was reported that the presence of an ortho-dihydroxyl group in the B ring of flavonoids is recognized as being basically important for achieving a high level of radical scavenging activity. There was further report about the relationship between scavenging ability and the structure of tea catechins (Nanjo *et al*., 1996). The scavenging effect of catechin was reduced drastically by the acetylation of hydroxyl groups in the B ring (3′, 4′-OH) whereas there was hardly any decrease in effectiveness with the modification of hydroxyl groups in the A ring (5, 7- or 3, 5, 7-OH). Thus, it is suggested that the A ring does not function as an advantageous structure to radical scavenging, and that the presence of hydroxyl groups in the B ring is the important structural feature for radical scavenging ability.

The overall result demonstrated that CGTase from *Paenibacillus* sp. RB01 can be used to synthesize epicatechin glucosides which were consisted of two major (Rt 48 and 45 min) and two minor products (Rt 30 and 27 min). After optimization, the yield of total products was 18.07%. The main product at Rt 48 min was a monoglucoside with higher solubility and browning resistance to UV irradiation than epicatechin while antioxidant property was slightly decreased. However, the product at Rt 45 min, a diglucoside structure, had lower solubility and browning resistance than the monoglucoside. The actual explanation of diglucoside properties was still unclear. For continual work, the minor product, especially the product at Rt 30 min which had slightly lower % yield than Rt 45 min will be further characterized.

CHAPTER V

CONCLUSIONS

1. Using starch adsorption, the purification of CGTase from *Paenibacillus* sp. RB01 was increased by 14.6 fold and a 69.8 percent yield was obtained.
2. The acceptor specificity of CGTase to catechins acceptors was in the following order: epigallocatechin gallate (EGCg) > epicatechin (EC) ≈ catechin (C).
3. The three substrates: beta-CD, starch and maltoheptaose were equally effective as glucosyl donor for this CGTase.
4. The optimum condition for transglucosylation by CGTase was: incubation of 80U/ml of CGTase with 1.0% beta-CD and 0.5% epicatechin in 10 mM acetate buffer pH 6.0 at 50 ºC for 24 hours. The 18.07% of total transglucosylation yield was obtained and consisted of the products at Rt 27 min 1.44%, Rt 30 min 3.39%, Rt 45 min 4.18% and Rt 48 min 8.33%, respectively.
5. Epimerization of epicatechin to catechin was observed and increased at alkaline pH, high temperature and longer incubation time.
6. From larger scale production, the 5.6% yield of the major product at Rt48 min was obtained.
7. The two major glucoside products: the Rt 48 and 45 min were identified as epicatechin-3′-*O*-α-D-glucopyranoside and epicatechin-3′-*O*-α-D-diglucopyranoside, respectively.
8. The solubility of epicatechin monoglucoside and diglucoside were 19 and 0.4 times higher than that of epicatechin.
9. The browning resistance to UV irradiation of epicatechin monoglucoside was higher than epicatechin diglucoside and epicatechin, respectively.
10. The IC_{50} of epicatechin, epicatechin monoglucoside and epicatechin diglucoside were 25, 36 and 46 μM, respectively.

REFERENCES

Abe, N., Nemoto, A., Tsuchiya, Y., Hojo, H., and Hirota, A. (2000). Studies on 1,1-diphenyl-2-picrylhydrazyl radical scavenging mechanism of a 2-pyrone compound. *Biosci Biotechnol Biochem* 64, 306-313.

Aga, H., Yoneyama, M., Sakai, S., and Yamamoto, I. (1991). Synthesis of 2-*0*-α-D-glucopyranosyl l-ascorbic acid by cyclomaltodextrin glucanotransferase from *Bacillus stearothermophilus. Agric Biol Chem* 55, 7, 1751-1756.

Amarowicz, R., and Shahidi, F. (1995). Antioxidant activity of green tea catechins in beta-carotene-linoleate model system. *J Food Lipids* 2, 47-56.

Aramsangtienchai, P., Chavasiri, W., Ito, K., Iizuka, M., and Pongsawasdi, P. (2007). Formation of epicatechin glucosides by cyclodextrin glycosyltransferase. In Kano, K. (eds.), *Proceedings of Fourth Asian Cyclodextrin Conference* pp.118-123.

Balentine, D.A., Wiseman, S.A., Bouwens, L.C. (1997). The chemistry of tea flavonoids. *Crit Rev Food Sci Nutr* 37, 693-774.

Bars, W., Heller, W., Michel, C., and Saran, M. (1990) Flavonoids as antioxidants: determination of radical-scavenging efficiencies. *Methods Enzymol* 186, 343-355

Beecher, G.R. (2003). Overview of dietary flavonoids: nomenclature, occurrence and intake. *J Nutr* 133, 3248S-3254S.

Belinky, P.A., Aviram, M., Mahmood, S., and Vaya, J. (1998). Structural aspects of the inhibitory effect of glabridin on LDL oxidation. *Free Radic Biol Med* 24, 1419-1429.

Bender, H. (1977). Cyclodextrin glucanotransferase from *Klebsiella pneumoniae* I. Formation, purification and properties of the enzyme from *Klebsiella pneumoniae* M 5 al. *Arch Microbiol* 111, 271-282.

Bidlack, W.R. (2001). Green Tea: health Benefits and Applications. *J Am Coll Nutr* 20, 656.

Birch, A.J., Clark-Lewis, J.W., and Robertson, A.V. (1957). The relative and absolute configurations of catechins and epicatechins. *J Chem Soc*, 3586-3593.

Bollag, M.D., and Edelstein, J.S. (1996). Protein Methods. New York: John Wiley & Sons, pp. 95-142.

Bradfield, A.E., and Penney, M. (1948). The catechins of green tea. Part 2, *J Chem Soc*, 2249-2254.

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

Bravo, L. (1998). Polyphenols: chemistry dietary sources metabolism and nutritional significance. *Nutr Rev* 56, 11, 317-333.

Chu, D.C. (1997). Green tea- its cultivation, processing of the leaves for drinking materials and kinds of green tea, in *Chemistry and applications of green tea* . Boca Raton, CRC Press pp 1.

Chu, D.C., and Juneja, L.R. (1997). General chemical composition of green tea and its infusion, in *Chemistry and applications of green tea.* Boca Raton, CRC Press pp.13.

Clifford, M. (2001). A nomenclature for phenols with special reference to tea. *Crit Rev Food Sci Nutr* 41(Suppl.), 393-397.

Cyclodextrin Technologies Development, Inc. (1990). Natural Cyclodextrins. Available from: http://www.cyclodex.com/about.asp?page_id=2&n=2 [7 June 2007].

El-Hady D.A. (2007). Selective and sensitive hydroxypropyl-beta-cyclodextrin based sensor for simple monitoring of (+)-catechin in some commercial drinks and biological fluids. *Anal Chem Acta* 593, 178–187.

Friedrich, W., and Galensa, R. (2002). Identification of a new flavonol glucoside from barley (*Hordeum vulgare* L.) and malt. *Eur Food Res Technol* 214, 388-393.

Funayama, M., Nishimo, T., Hirota, A., Murao, S., Takenishi, S. and Nakano, H. (1993). Enzymatic synthesis of (+)-catechin-α-glucoside and its effect on tyrosinase activity. *Biosci Biotech Biochem* 57, 1666-1669.

Fuwa, H. (1954). A new method for micro determination of amylase activity by the use of amylose as substrate. *J Biochem* 41, 583-603.

Gawande, B. N., Goel, A., Patkar, A. Y., and Nene, S. N. (1999). Purification and properties of a novel raw starch degrading cyclomaltodextrin

glucanotransferase from *Bacillus firmus*. *Appl Microbiol Biotechnol* 51, 504-509.

Goel, A., and Nene, S.N. (1995). Modifications in the phenolphthalein method for spectrophotometric estimation of beta-cyclodextrin. *Starch-Starke* 399-400.

GE Healthcare (2006). Sephadex LH-20. Available from: http://www4.gelifesciences.com/aptrix/upp00919.nsf/Content/130351BF947E62E2C1256EB40044ACD8/$file/56119097AD.pdf [1 July 2006].

Hashimoto, F., Nonaka, G.I., and Nishioka, I. (1988). Tannins and related compounds. LXIX. Isolation and structure elucidation of B, B′-linked bisflavonoid, theasinensins D-G and ooolongtheanin from oolong tea. *Chem Pharm Bull* 36, 5, 1676-1684.

Hara, Y., Matsuzaki, T., and Suzuki, T. (1987). Angiotensin I converting enzyme inhibiting activity of tea components. *J Agric Chem Soc Jpn* 61, 803-808.

Hara, Y., and Watanabe, M. (1989). Antibacterial activity of tea polyphenols against *Clostridium botulinum. Nippon Shokuhin Kogyo Gakkaishi* 36, 951-955.

Hergert, H.L., and Kurth, E.T. (1953). The isolation and properties of catechol from white fir bark. *J Org Chem* 18, 521-529.

Hirose, M., Hoshiya, T., Akagi, K., Futakuchi, M., and Ito, N. (1994). Inhibition of mammary gland carcinogenesis by green tea catechins and other naturally occurring antioxidants in female Sprague-Dawley rats pretreated with 7, 12-dimethylbenz[a]antracene. *Cancer Lett* 83, 149-156.

Horikoshi, K. (1971). Production of alkaline enzymes by alkalophilic microorganisms. *J Agr Biol Chem* 35, 1783-1791.

Ikeda, T. (2007). Sugar substitutes: reasons and indications for their use. Available from: http://www.level1diet.com/research/ id/211349 [4 September 2007].

Ishizu, T., Kintsu, K., and Yamamoto, H. (1999). NMR study of the solution structures of the inclusion complexes of β-cyclodextrin with (+)-catechin and (−)-epicatechin. *J Phys Chem* 103, 8992–8997.

Kada, T., Kaneko, K., Matsuzaki, S., and Matsuzaki, T. (1985). Detection and chemical identification of natural biomutagens: a case of green tea factor. *Mutation Res* 150, 127-132.

Kandaswaani, C., and Middleton, E. (1994). Free radical scavenging and antioxidant activity of plant flavonoids. *Adv Exp Med Biol* 336, 351-376.

Kaskangam, K. (1998). *Isolation and characterization of cyclodextrin glycosyltransferase isozymes from Bacillus sp. A11.* Master's Thesis Biochemistry, Faculty of Science, Chulalongkorn University.

Kato, T., Horikoshi, K. (1986). Cloning and expression of the *Bacillus subtilis* No. 313 γ-cyclodextrin forming CGTase gene in *Escherichia coli*. *Agric Biol Chem* 50, 2161-2162.

Kitahata, S., Tsuyama, N., and Okada, S. (1974). Purification and some properties of cyclodextrin glycosyltransferase form a strain of *Bacillus* species. *Agric Biol Chem* 38, 387-393.

Kitahata, S. (2000). Studies on the development of functional oligosaccharides Using Amylases and Related Enzymes. *J Appl Glycosci* 47, 1, 87-97.

Kitao, S., Ariga, T., Matsudo, T. and Sekine, H. (1993). The syntheses of catechin-glycosides by transglycosylation with *Leuconostoc mesenteroids* sucrose phosphorylase. *Biosci Biotech Biochem* 57, 2010-2015.

Koketsu, M. (1997). Antioxidative activity of tea polyphenols, in *Chemistry and applications of green tea.* Boca Raton, CRC Press pp. 37-42.

Kometani, T., Terada, Y., Nishimura, T., Taki, Hiroshi T., and Shigataka, O. (1994). Transglycosylation to hesperidin by glucanotransferase from alkalophilic *Bacillus* species in alkaline pH and properties of hesperidin glycosides. *Biosci Biotec Biochem* 58, 11, 1990-1994

Laloknam, S. (1997). *Detection of cyclodextrin glyclosyltransferase gene by synthetic oligonucleotide probes*. Master's Thesis Biochemistry, Faculty of Science, Chulalongkorn University.

Li, D.L., Li, X.M., Peng, Z.Y., and Wang, B.G. (2007). Flavanol derivatives from *Rhizophora stylosa* and their DPPH radical scavenging activity. *Molecules 12*, 1163-1169.

Martín, M. T., Cruces, M.A., Alcalde, M., Plou, F. J., Bernabe, M.., Ballesteros, A. (2004). Synthesis of maltooligosyl fructofuranosides catalyzed by immobilized cyclodextrin glucosyltransferase using starch as donor. *Tetrahedron 60*, 529–534.

Matsuzaki, T., and Hara, Y. (1985). Antioxidative activity of tea leaf catechins. *Nippon Nogeik Kaishi* 59, 129-134.

Mayer, J. (2006). Health benefits of green Tea. Available from:

http://www.e-clipse.com/green-tea.htm [3 June 2007].

Meager, M.M., Nikolov, Z.L., and Reilly, P.J. (1989). Subsite mapping of *Aspergillus niger* glucoamylase I and II. *Biotechnol Bioeng* 34, 681–688.

Meulenbeld, H. G., Zuilhof, H., Veldhuizen, A., Heuvel, R., and Hartmans, S. (1999). Enhanced (+)-catechin transglucosylating activity of *Streptococcus mutans* GS-5 glucosyltransferase-D due to fructose removal. *Appl Env Micro* 65, 9, 4141-4147.

Miranda, C., and Buhler, D. R. (2000). Antioxidant activities of flavonoids. Available from: http://lpi.oregonstate.edu/f-w00/flavonoid.html [14 May 2007].

Miliauskasa, G., Venskutonis, P.R., and van Beek, T.A. (2004) Screening of radical scavenging activity of some medicinal and aromatic plant extracts**.** *Food Chem* 85 231–237**.**

Mojsił, J. (1999). Flavonoids and their gastroprotective effect. (in Slovak), *Cs Slov Gastroenterol* 53, 170-174.

Mojsilove, G., and Kuchta, M. (2001). Dietary flavonoids and risk of coronary heart disease. *Physiol Res* 50, 529-535.

Magomet, M., Tomov, T., Somann, T., and Abelyan, V. H. (2005). Sweetener and use. Available from: http://www.freshpatents.com/ Sweetner-and-use-dt20070412ptan20070082102.php [5 September 2007].

Monach, C., Scalbert, A., Morand, C., Rémésy, C., and Jiménez, L. (2004). Polyphenols: food sources and bioavailability. *Am J Clin Nutr* 79, 727-47.

Moon, Y.H., Kim, Ghahyun., Lee, J.H., Jin, X.J., Kim, D.W., and Kim, D. (2006). Enzymatic synthesis and characterization of novel epigallocatechin gallate glucosides. *J Mol Anal* 40, 1-7.

Muramatsu, K., Fukuyo, M., and Hara, Y. (1986). Effect of green tea catechins on plasma cholesterol level in cholesterol-fed rats. *J Nutr Sci Vitaminol* 32, 613-622.

Nagai, T., Miyaichi, Y., Tomimori, T., Suzuki, Y., and Yamaha, H. (1992). Anti-viral activity of two flavonoids from Tanacetum micro-phyllum. *Antiviral Res* 19, 207-216.

Nakabayashi, T. (1991). Chemical components in tea leaves, in *Chemistry and function of green tea, black tea, and oolong tea.* Boca Raton, CRC Press pp. 20.

Nakahara, K., Kontari, M., Ono, H., Kodama, T., Tanaka, T., Ooshima, T., and Hamada S. (1995). Glucosyltransferase from *Streptococcus sobrinus* catalyzes glucosylation of catechin. *Appl Environ Microb* 61, 7, 2768–2770.

Nakane, H., and Ono, K. (1990). Differential inhibitory effects of some catechins derivatives on the activities of HIV reverse transcriptase and cellular deoxyribonucleic acid and RNA polymerase. *Biochemistry* 29, 2841-2845.

Namiki, K., Yamanaka, M., Tateyama, C., Igarashi, M., and Namiki, M. (1991). Platelet aggregation inhibitory activity of tea extracts. *Nippon Shokuhin Kogyo Gakkaishi* 38, 3, 189-195.

Ninomiya, M., Unten, L., and Kim, M. (1997). Chemical and physicochemical properties of green tea polyphenols, in *Chemistry and applications of green tea.* Boca Raton, CRC Press. pp 23.

Nishimura, T., Kometani, T.,Takii, H., Terada, Y., and Okada, S. (1994). Acceptor specificity in the glucosylating reaction of *Bacillus subtilis* X-23 α-amylase towards various phenolic compounds and the structure of kojic acid glucoside. *J Ferment Bioeng* 78, 37-41.

Park, D.C., Kim, T.K., Lee, Y.H. (1998). Characteristics of transglycosylation reaction of cyclodextrin glucanotransferase in the heterogeneous enzyme reaction system using extrusion starch as a glucosyl donor. *Enzyme Microb Technol* 22, 217-222.

Prousoontorn, M. H., and Pantatan, S. (2007). Production of 2-*O*-a-glucopyranosyl L-ascorbic acid from ascorbic acid and β-cyclodextrin using immobilized cyclodextrin glycosyltransferase. *J Incl Phenom Macrocycl Chem* 57, 39–46.

Read, M.A. (1995). Flavonoids: naturally occurring antiinflamatory agents. *Am J Pathol* 147**,** 235-237.

Rice-Evans, C.A., Miller, N.J., Bolwell, P.G., Bramley, P.M., and Pridham, J.B. (1995). The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radical Res* 22, 4, 375–383.

Rutchtorn, U. (1993). *Production of cyclodextrin glucanotransferase in fermenter and its immobilized on DEAE-cellulose.* Master's Thesis Biochemistry, Faculty of Science, Chulalongkorn University.

Sakanaka, S., Sato, T., Kim, M., and Yamamoto, T. (1990). Inhibitory effects of green

tea polyphenols on glucan synthesis and cellular adherance of cariogenic streptococci. *Agric Biol Chem* 54, 2925-2929.

Sato,T., Nakagawa, H., Kurosu, J., Yoshida, K., Tsugane, T., Shimura, S., Kirimura, K., Kino, K., and Usami, S. (2000). α-Anomer-selective glucosylation of (+)-catechin by the crude enzyme, showing glucosyl transfer activity, of *Xanthomonas campestris* WU-9701. *J Biosci Bioeng* 90, 6, 625-630.

Schmid, G. (1989). Cyclodextrin glucanotransferase production: yield enhancement by overproduction of cloned gene. *Trends Biotechnol* 7, 244-248.

Silvina, B. L., and Cesar, G. F. (2000). Ascorbate protects (+)-catechin from oxidation both in a pure chemical system and human plasma. *Biol Res* 33, 151-157.

Stagg, G.V., Millin, D.J. (1975). The nutritional and therapeutic value of tea-a review. *J Sci Food Agric* 26, 1439–1459.

Stevian Biotechnology (2007). What is stevia? Available from: http://www.em-x.net/stevia.html. [4 September 2007].

Szejtli, J. (1988). *Cyclodextrin technology* Kluwer Academic Publisher Dordrecht. pp.450.

Szejtli, J. (2004). Past, present, and future of cyclodextrin research. *Pure Appl Chem* 76, 10, 1825-1845.

Techaiyakul, W. (1991). *Production and characterization of cyclodextrins glucanotransferase from Bacillus* sp. Master's Thesis Biochemistry, Faculty of Science, Chulalongkorn University.

Terao, J., Piskula, M., and Yao, Q. (1994). Protective effect of epicatechin gallate, and quercetin on lipid peroxidation in phospholipid bilayers. *Arch Biochem Biophys* 308, 278-284.

Tesana, S. (2001). *Cyclodextrin glycosyltransferase from thermotolerant bacteria: screening, optimization, partial purification and characterization.* Master's Thesis Biochemistry, Faculty of Science, Chulalongkorn University.

Tonkova, A. (1998). Bacterial cyclodextrin glucanotransferase. *Enzyme Microb Tech* 22, 678-686.

Tsuchiya, H. (2001). Stereospecificity in membrane effects of catechins. *Chem Biol Interact* 134, 41–54.

United States Department of Agriculture (2003). USDA database for the flavonoid content of selected foods -2003. Available from: http://www.nal.usda.gov/fnic/foodcomp/Data/Flav/flav.html [14 May 2007].

Van der Veen, B. A., Uitdehaag, J. C. M., Penninga, D., Van Alebeek, G. J. W. M., Smith, L. M., Dijkstra, B. W., and Dijkhuizen, L. (2000). Rational design of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251 to increase alpha-cyclodextrin production. *J Mol Biol* 296, 1027-1038.

Wall, P.E. (2005). *Thin-layer chromatography: a modern practical approach*. RSC Publishing pp.140

Xu, J.Z., Leung, L.K., Huang, Y., and Chen, Z.Y. (2003). Epimerisation of tea polyphenols in tea drinks. *J Sci Food Agr* 83, 15, 1617-1621.

Yamamura, S., Ozawa, K., Ohtanik, K., Kasai, R., and Yamasaki, K. (1998). Antihistaminic flavones and aliphatic glycosides from Mentha spicata. *Phytochemistry* 48, 131-136.

Yenpetch, W. (2002). *Purification and biochemical characterization of cyclodextrin glycosyltrasferase from thermotolerant Paenibacillus sp. strain* RB01. Master's Thesis Biochemistry, Faculty of Science, Chulalongkorn University.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

APPENDICES

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Appendix 1 Preparation for polyacrylamide gel electrophoresis

1) Stock reagents

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

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

Adjusted volume to 100 ml with distilled water

1.5 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane	18.17 g

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

2 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane	24.2 g

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

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 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	21 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)

10.0 % separating gel

30% Acrylamide solution	2.50 ml
Solution B (SDS-PAGE)	2.50 ml
Distilled water	2.39 ml
10% $(NH_4)_2S_2O_8$	100 µl
TEMED	10 µl

5.0% stacking gel

30% Acrylamide solution	0.84 ml
Solution C (SDS-PAGE)	1.0 ml
Distilled water	3.1 ml
10% $(NH_4)_2S_2O_8$	50 µl
TEMED	10 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 (hydroxylmethyl)-aminometane	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 2 Preparation for buffer solution

0.2 M Sodium Acetate pH 4.0, 5.0 and 6.0

CH_3COONa	1.21 g

Adjusted volume to 100 ml with distilled water. Adjusted to pH 4, 5 or 6 by 0.2 M acetic acid

0.2 M Phosphate pH 6.0

KH_2PO_4	3.28 g
K_2HPO_4	0.16 g
Distilled water	100 ml

0.2 M Phosphate pH 7.0

KH_2PO_4	1.35 g
K_2HPO_4	1.67 g
Distilled water	100 ml

0.2 M Phosphate pH 8.0

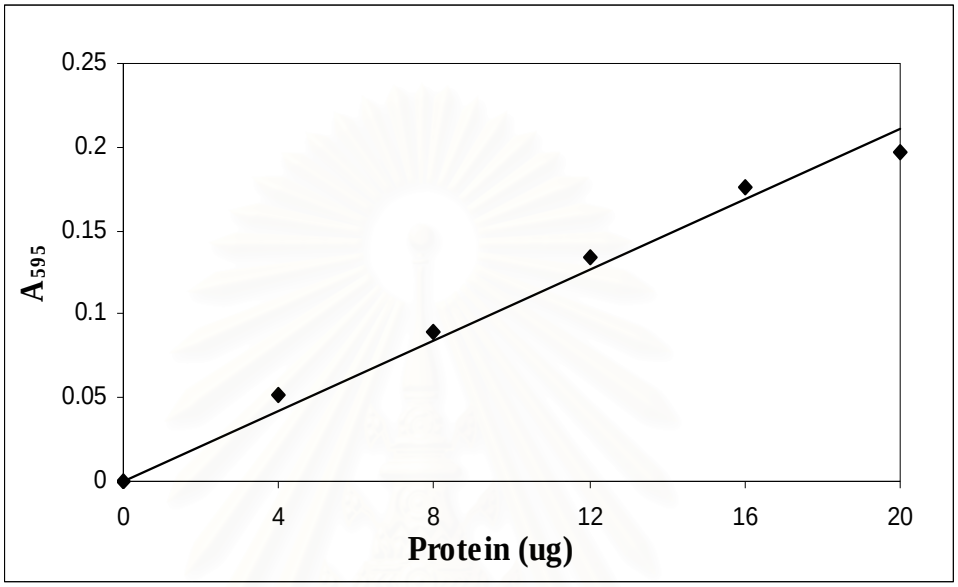
KH_2PO_4	0.48 g
K_2HPO_4	2.34 g
Distilled water	100 ml

0.2 M Tris-Glycine NaOH pH 8.0, 9.0 and 10.0

glycine	1.5 g

Adjusted to pH 8.0, 9.0 and 10.0 by 1 M NaOH and adjusted volume to 100 ml with distilled water.

Appendix 3 Standard curve for protein determination by Bradford's method



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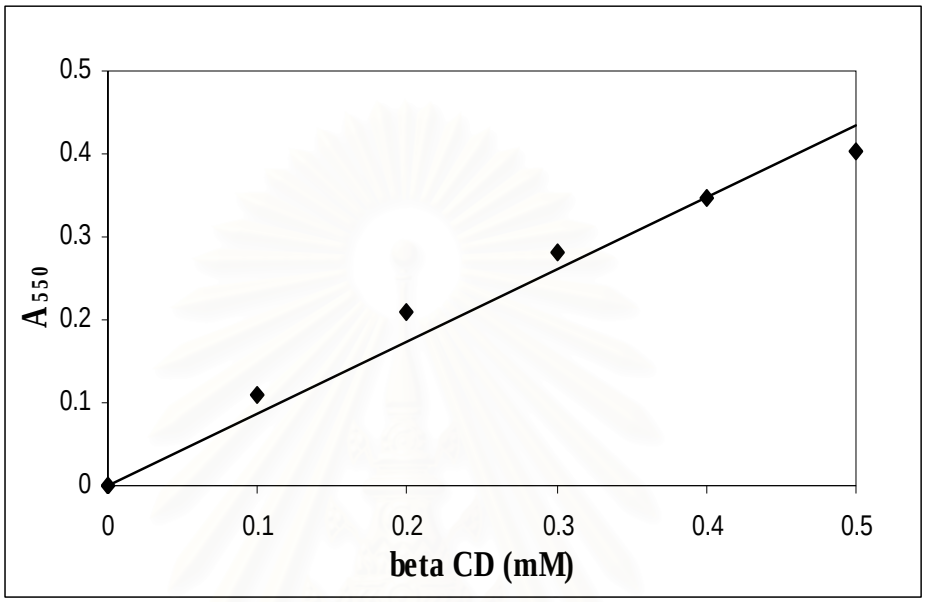
จุฬาลงกรณ์มหาวิทยาลัย

Appendix 4 Standard curve of beta–cyclodextrin by phenolpthalein method



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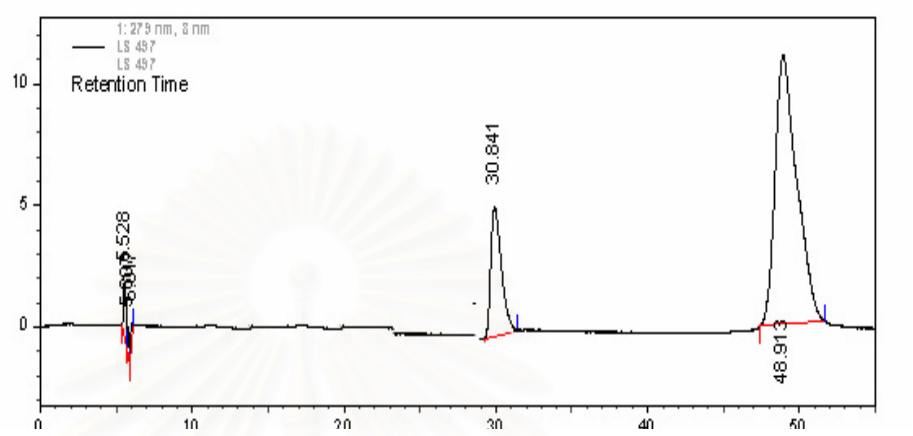
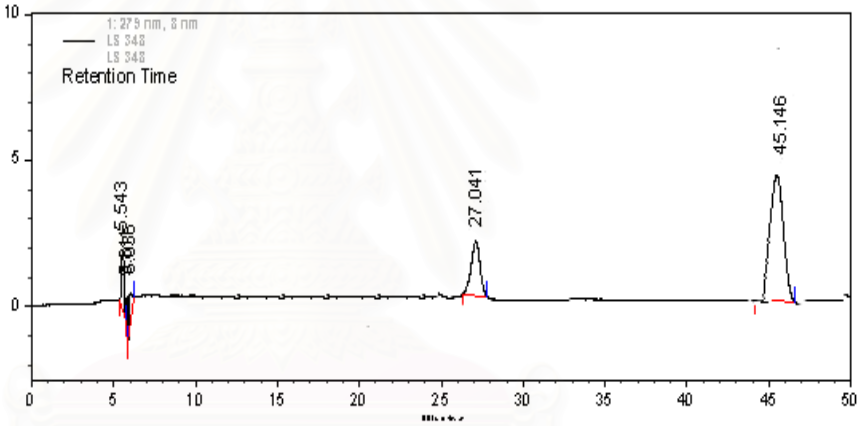
Appendix 5 "Glu I" and "Glu II" by HPLC

"Glu I"



"Glu II"



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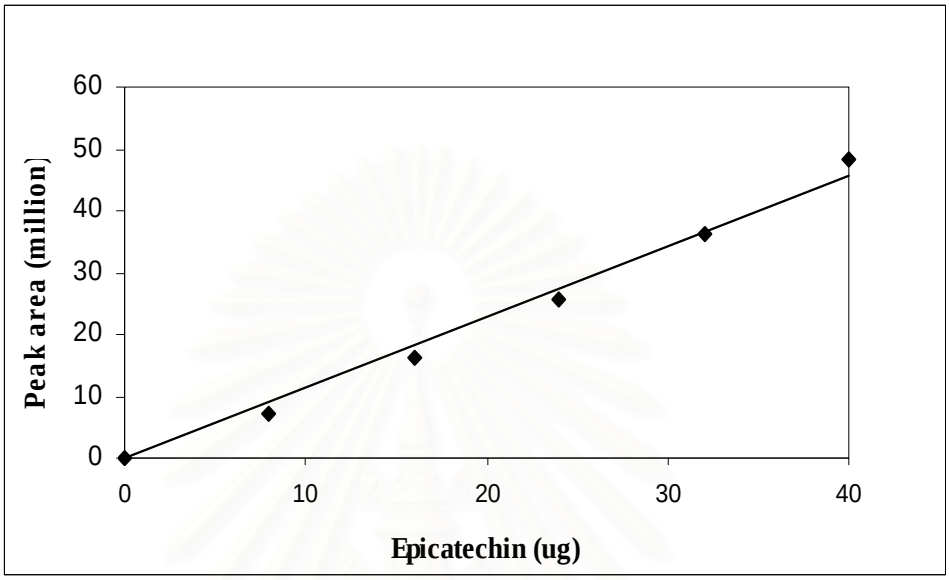
Appendix 6 Standard curve of epicatechin by HPLC



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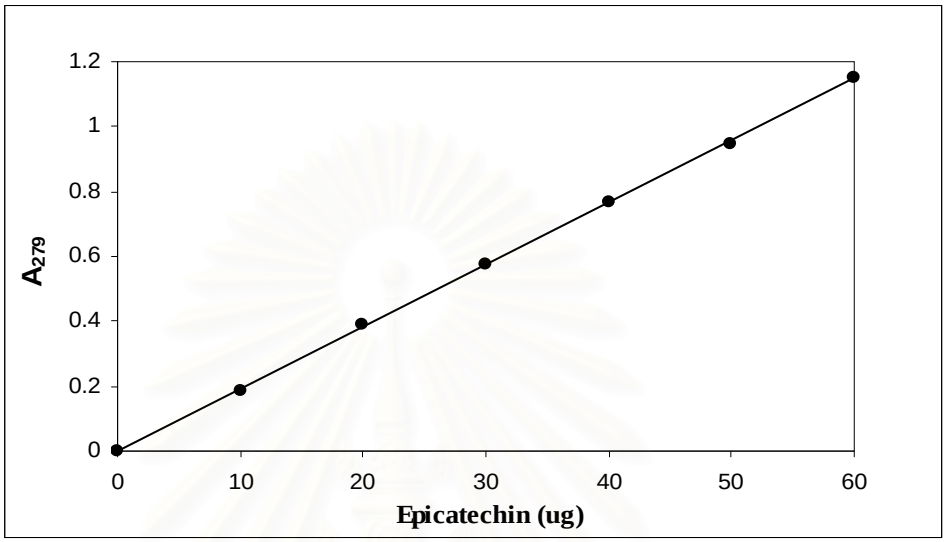
Appendix 7 Standard curve of epicatechin by UV-spectrophotometry



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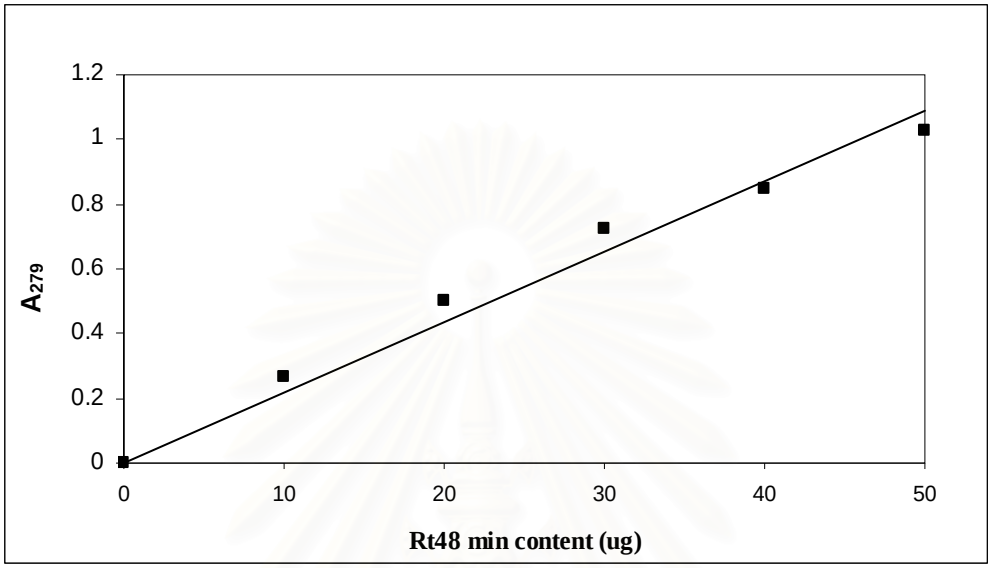
Appendix 8 Standard curve of the product at Rt 48 min by UV-spectrophotometry



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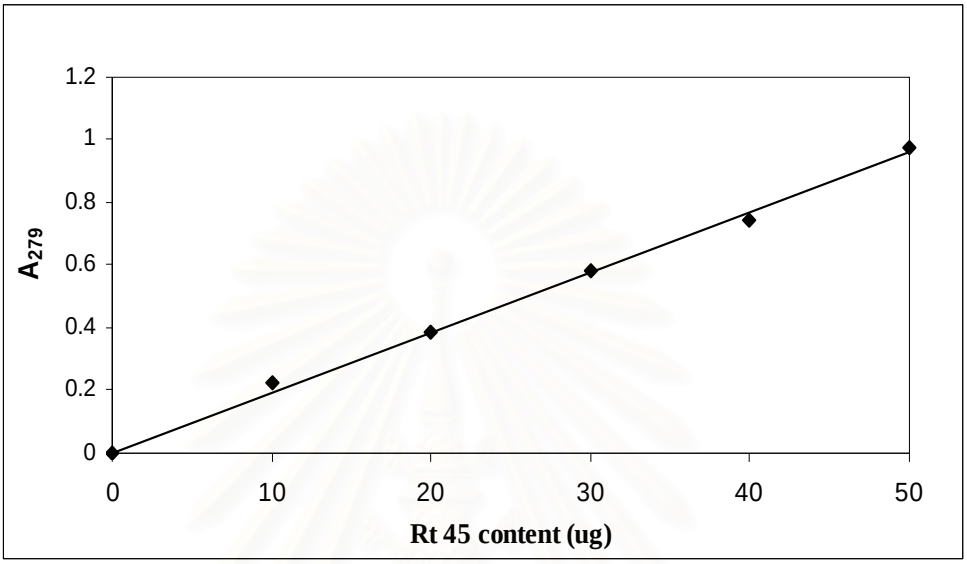
Appendix 9 Standard curve of the product at Rt 45 min by UV-spectrophotometry



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BIOGRAPHY

Miss Pornpun Aramsangtienchai was born on September 6, 1982. She graduated with the Bachelor's degree of Science in Biochemistry program from Chulalongkorn University in 2005, and continued studying for the Master degree of Science in Biochemistry program.

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