การสังเคราะห์วิตามินอีไกลโคไซด์โดยแอลฟากลูโคซิเดสจากยีสต์ *Saccharomyces cerevisiae* และไซโคลเดกซ์ทรินไกลโคซิลทรานสเฟอเรสจาก *Paenibacillus* sp. RB01

นายพิษณุ ธนดลเสถียร

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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SYNTHESIS OF VITAMIN E GLYCOSIDE BY ALPHA-GLUCOSIDASE FROM *SACCHAROMYCES CEREVISIAE* AND CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM *PAENIBACILLUS* SP. RB01

Mr. Pisanu Thanadolsathien

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biotechnology
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สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Thesis Title	SYNTHESIS OF VITAMIN E GLYCOSIDE BY ALPHA-GLUCOSIDASE FROM *SACCHAROMYCES CEREVISIAE* AND CYCLODEXTRIN GLYCOSYL TRANSFERASE FROM *PAENIBACILLUS* sp. RB01
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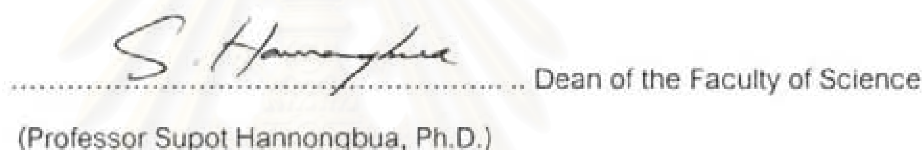
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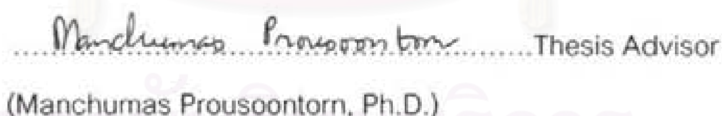
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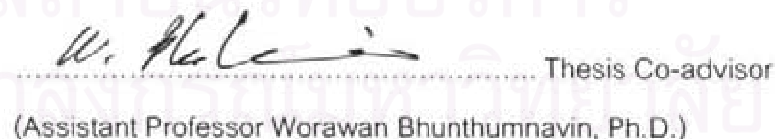
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พิชณุ ธนดลเสถียร : การสังเคราะห์วิตามินอีไกลโคไซด์โดยแอลฟากลูโคซิเดสจากยีสต์ *Saccharomyces cerevisiae* และไซโคลเดกซ์ทรินไกลโคซิลทรานสเฟอเรสจาก *Paenibacillus* sp. RB01 (SYNTHESIS OF VITAMIN E GLYCOSIDE BY ALPHA-GLUCOSIDASE FROM *SACCHAROMYCES CEREVISIAE* AND CYCLODEXTRIN GLYCOSYL TRANSFERASE FROM *PAENIBACILLUS* sp. RB01) อ. ที่ปรึกษา : อ.ดร.มัญชุมาส เพราะสุนทร, อ.ที่ปรึกษาร่วม : ผศ.ดร.วรวรรณ พันธุมนาวิน 110 หน้า.

การสังเคราะห์วิตามินอีไกลโคไซด์ตัวใหม่โดยอาศัยปฏิกิริยาการโยกย้ายหมู่ไกลโคซิลสองขั้นตอนของแอลฟากลูโคซิเดสจาก *Saccharomyces cerevisiae* และไซโคลเดกซ์ทรินไกลโคซิลทรานสเฟอเรส (CGTase) จาก *Paenibacillus* sp.RB01 ในขั้นแรกเป็นการสังเคราะห์ 2-(α-D-glucopyranosyl)methyl-2,5,7,8-tetramethylchroman-6-ol (TMG) จาก 2-hydroxyl-2,5,7,8-tetramethylchroman-6-ol (TM) และน้ำตาลมอลโทสโดยแอลฟา-กลูโคซิเดส จากนั้นในขั้นที่สองเป็นการนำ TMG มาเป็นสารตั้งต้นในการสังเคราะห์อนุพันธ์ของวิตามินอีตัวใหม่ โดยใช้ CGTase ในการโยกย้ายหมู่กลูโคซิลจากบีตา-ไซโคลเดกซ์ทรินให้กับ TMG พบว่าผลิตภัณฑ์ไกลโคไซด์ตัวใหม่ มีโครงสร้างเป็น α-D-glucopyranosyl-α(1→4)-2-(α-D-glucopyranosyl) methyl-2,5,7,8-tetramethylchroman-6-ol (TMG_2) จากการวิเคราะห์ด้วยเทคนิค mass spectrometry และ nuclear magnetic resonance spectroscopy เมื่อทำการบ่ม TMG เข้มข้น 0.5% (w/v) และบีตาไซโคลเดกซ์ทรินเข้มข้น 1.5% (w/v) กับ CGTase ปริมาณ 100 ยูนิตในบัฟเฟอร์โพแทสเซียมฟอสเฟต 50 มิลลิโมลาร์ที่อุณหภูมิ 50 $^{\circ}$ซ เป็นเวลา 4 ชั่วโมง พบว่าผลผลิตที่ได้คิดเป็นเปอร์เซ็นต์ของ TMG_2 เทียบกับปริมาณ TMG ที่ให้ไปเท่ากับ 47.2% การละลายน้ำของ TMG_2 สูงกว่า TMG 14 เท่าและสูงกว่า TM และ Trolox มากกว่า 1×10^4 และ 7×10^4 เท่าตามลำดับ โดยที่ความสามารถในการกำจัดอนุมูลอิสระ 1,1-diphenyl-2-picrylhydrazyl (DPPH) ของ TMG_2 ใกล้เคียงกับ TMG, TM และ Trolox

หลักสูตร.........เทคโนโลยีชีวภาพ.............ลายมือชื่อนิสิต.....พิชณุ ธนดลเสถียร.....

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PISANU THANADOLSATHIEN : SYNTHESIS OF VITAMIN E GLYCOSIDE BY ALPHA-GLUCOSIDASE FROM *SACCHAROMYCES CEREVISIAE* AND CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM *PAENIBACILLUS* sp. RB01. THESIS ADVISOR: MANCHUMAS PROUSOONTORN, Ph.D. THESIS CO-ADVISOR : ASST.PROF. WORAWAN BHUNTHUMNAVIN, Ph.D. 110 pp.

A novel vitamin E glycoside was synthesized by a two-step enzymatic transglycosylation system, a combination of α-glucosidase from *Saccharomyces cerevisiae* and cyclodextrin glycosyltransferase (CGTase) from *Paenibacillus* sp. RB01. 2-(α-D-Glucopyranosyl)methyl-2,5,7,8-tetramethylchroman-6-ol (TMG) was synthesized from 2-hydroxyl-2,5,7,8-tetramethylchroman-6-ol (TM) and maltose by α-glucosidase. TMG was then used as an acceptor to synthesize a novel vitamin E derivative by CGTase using β-cyclodextrin (β-CD) as a donor. A novel product was identified as α-D-glucopyranosyl-(1→4)-2-(α-D-glucopyranosyl)methyl-2,5,7,8-tetramethylchroman-6-ol (TMG_2) by mass spectrometry and nuclear magnetic resonance spectroscopy analysis. When 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% (w/v) of TMG, 1.5% (w/v) of β-CD and 100 U of CGTase was incubated at 50 $^{\circ}C$ for 4 hours, the percent yield of TMG_2 reached 47.2% based on the amount of TMG supplied. The solubility in water of TMG_2 was about 14 times higher than that of TMG and was more than 1×10^4 and 7×10^4 times higher than those of TM and Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), respectively. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) free-radical scavenging activity of TMG_2 was found to be nearly the same as those of TMG, TM and Trolox.

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ABBREVIATIONS

A	Absorbance
Å	Angstorm
BSA	Bovine serum albumin
CDs	Cyclodextrin
CGTase	Cyclodextrin glycosyltransferase
^oC	Degree Celsius
Da	Dalton
DPPH	2, 2-Diphenyl-1-picryhydrazyl
ESI-TOF-MS	Electrospray Ionization-Time of Flight
et al.	Et. Alii (latin),and others
G	Gram
H	Hour
HPLC	High Performance Liquid Chromatography
L	Liter
LDL	Low Density Lipoprotein
μg	Microgram
μl	Microliter
NMR	Nuclear Magnetic Resonance
M	Molar
Min	Minute
mL	Milliliter
MW	Molecular weight
PAGE	Polyacrylamide gel electrophoresis
R_f	Relative mobility
R_t	Retention time
SDS	Sodium dodecyl sulfate
U	Unit(s)
v/v	Volume by volume
Vmax	Maximum velocity
w/v	Weight by volume

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CHAPTER I

INTRODUCTION

1.1 Vitamin E

1.1.1 General information of vitamin E

Vitamin E is one of the most important lipid-soluble antioxidant compounds in the human cells. However, it can be exclusively obtained from the diet. “Vitamin E” is the term for a family of two groups of homologues – tocopherols and tocotrienols – that are synthesized by plants from homogentisic acid (Figure 1) (Christie, 2007). Vitamin E consists of two main components that are 6-chromanol ring (Chromanoxyl “Head” ring) and long phytyl “Tail” side chain (Figure 2) (Burton and Traber, 1990). Tocopherols have a saturated 16-carbon phytyl side chain. They differ from tocotrienols, which have three double bonds on their side chain (Figure 3) (FAO/WHO, 2002). However, there are only α-tocopherol and γ-tocopherol from all homologues that are used in the body. They are absorbed in the small intestine and transported via blood circulation to be accumulated in the liver and secreted to other cells for lipid metabolism (Figure 4). In addition, α-tocopherol in blood concentrations is usually 4-10 times higher than γ-tocopherol. As a result, it has been shown that α-tocopherol acts as the most important bioactive antioxidant *in vivo* (Frank, 2005). Some properties of α-tocopherol are shown in Table 1 (Kirahara, 2007 and Sciencelab, 2005).

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1.1.2 Antioxidant mechanism and applications of vitamin E

Mechanism of antioxidant activity of vitamin E is inhibition of lipid peroxidation resulting from free radicals, especially hydroxyl radical ($HO^{\bullet}$) and superoxide anion ($O_2^{\bullet-}$). Free radicals are highly reactive molecules or atoms that have an unpaired electron – free from making a bond with other molecules. They can easily damage

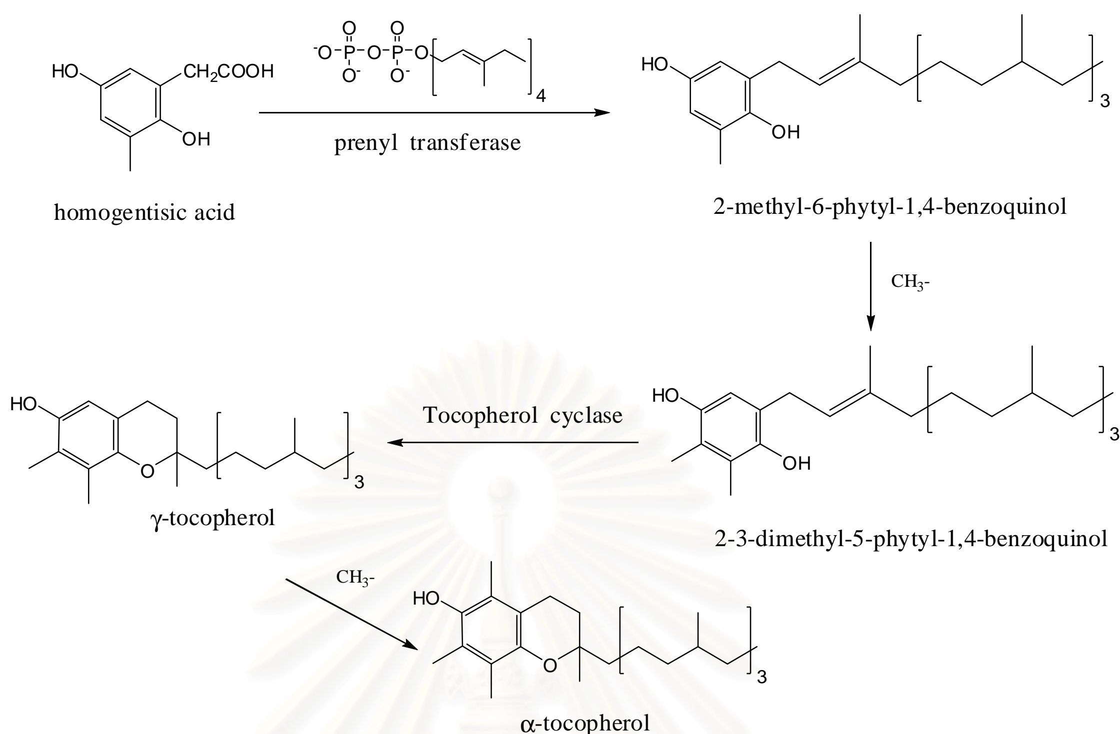

Figure 1. Biosynthesis of α-tocopherol in the plant chloroplast. Homogentisic acid is condensed with phytyl diphosphate via prenyl transferase-calatyzed reaction. Afterwards, intermediate is methylated and converted to γ-tocopherol by tocopherol cyclase. The last step is methylation of γ-tocopherol resulting in α-tocopherol (Christie, 2007).

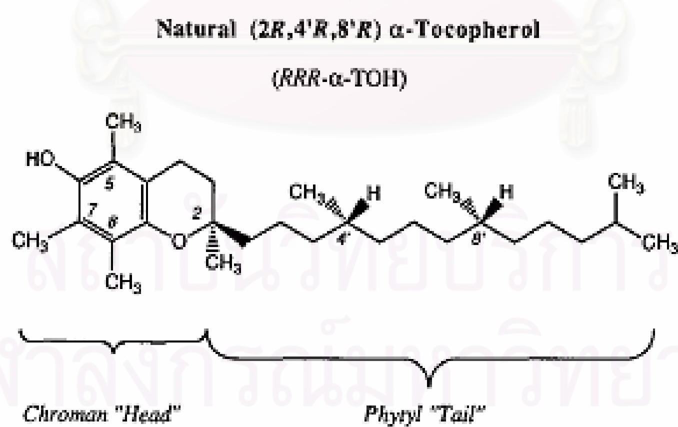

Figure 2. Structure of 2*R*,4'*R*,8'*R*-α-tocopherol (Burton and Traber, 1990).

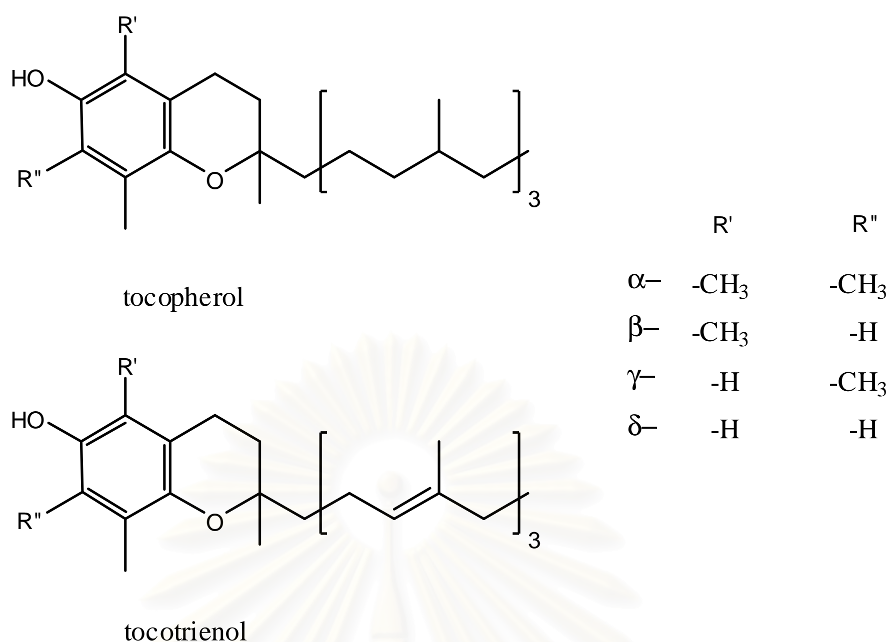

Figure 3. Tocopherol and tocotrienol homologues (Christie, 2007).

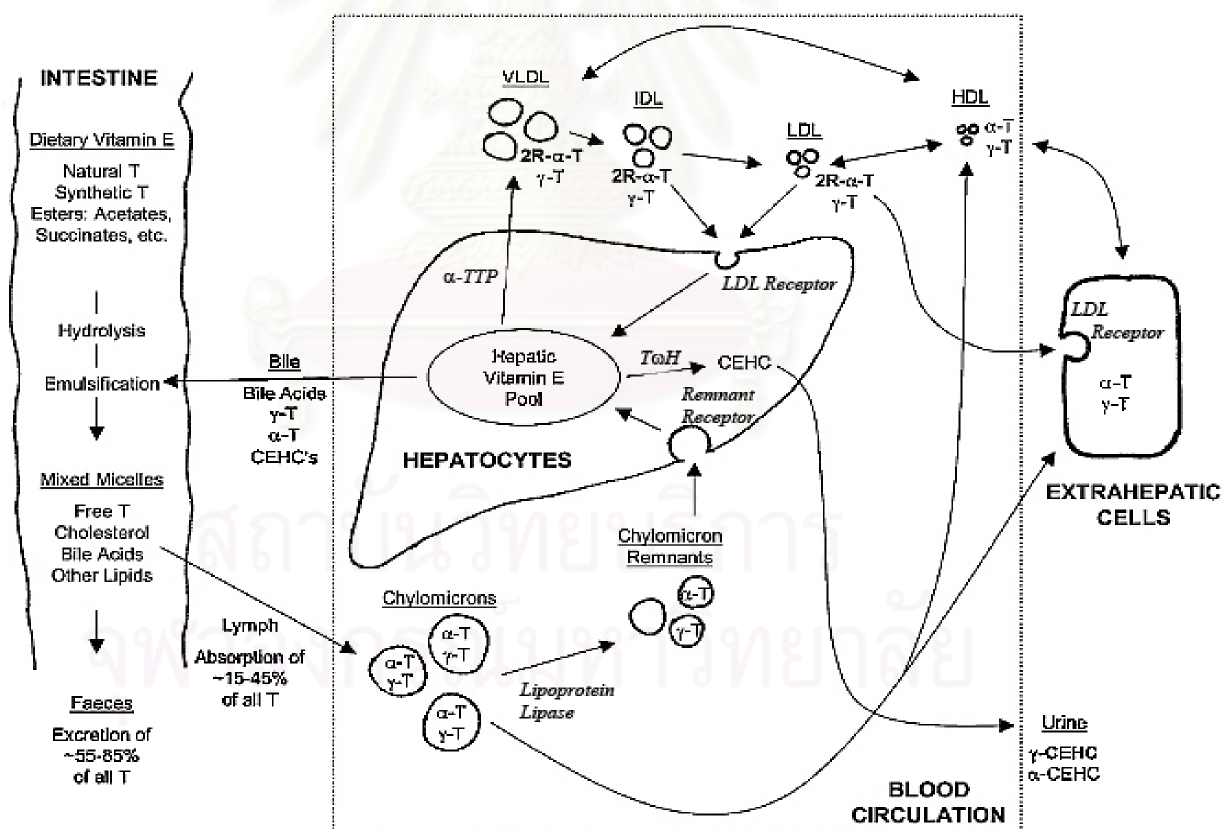

Figure 4. Absorption, transport and metabolism of α- and γ-tocopherol in the body. Abbreviation used: CEHC, carboxyethyl hydroxychroman metabolites; α-TTP, α-tocopherol transfer protein; T, tocopherol; TωH, tocopherol-ω-hydroxylase; VLDL, IDL, LDL and HDL; very low-, intermediate-, low-, high-density lipoproteins, respectively (Frank, 2005).

Table 1 Physical and chemical properties of α-tocopherol (Kirahara, 2007 and Sciencelab, 2005)

Property	Characteristics
Name	2*R*-(4'*R*,8'*R*,12'-trimethyltridecyl)-2,5,7,8-tetramethylchroman-6-ol
Formula	$C_{29}H_{50}O_2$
Molecular weight	430.706
Density	0.95
Physical state and appearance	Yellow, odorless, tasteless, oily and viscous liquid
Melting point	3 ^{O}C (37.4^{O}F)
Flash point	240°C (464°F) in closed cup
Refractive index	+0.32^{O} in ethanol, -3.0^{O} in benzene
Solubility	Practically insoluble in cold water Soluble in acetone, ethanol, ether, methylene chloride and fatty oils
Instability and Incompatibility	Instability in excess heat, light, air Incompatibility with oxidizing agents, metals, UV light, alkalies.
Corrosivity	Non-corrosive in presence of glass
Toxicity	Acute oral toxicity (LD_{50}): ~4000 mg/kg [mouse]

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proteins, lipids and any cell component with three steps of lipid peroxidation – initiation phase (polyunsaturated fatty acid transformation into carbon-centered radical by any radical generator), propagation phase (carbon-centered radical and molecular oxygen are fused together as highly reactive peroxyl radical ($ROO^{\bullet}$) and attack other polyunsaturated fatty acid that form another carbon-centered radical that cause a chain reaction) and termination stage (peroxyl radical reacts with another peroxyl radical giving inactive product) (Figure 5A) (Young and McEneny, 2001). Vitamin E has an effect on propagation phase by preventing polyunsaturated fatty acid from creating more free radicals by collision with peroxyl radical. For example, one molecule of α-tocopherol reacts with one unit of peroxyl radical resulting in a stable compound – α-tocoperoxyl radical (α-$TO^{\bullet}$). This causes the end of lipid peroxidation (Figure 5B) (Burton and Traber, 1990). Therefore, vitamin E can prevent cardiovascular disease by decreasing LDL oxidation. Moreover, it can reduce the risk of cancer by increasing natural killer cell activity (apoptosis) and reducing oxidative DNA damage in target cells. Furthermore, it has protective effect against damage caused by skin aging resulting from the augmentation of T-cell-mediated function (age-associated decreases) (Meydani, 1995). From these properties, vitamin E is applied as high-valued ingredients for various fields of manufacturing industries. For example, the pharmaceutical industry adds vitamin E to supplementary foods, medicine and feeds supplying to vitamin E-deficient patients, treating some diseases and feeding for livestock (Rodrigo *et al.*, 2007 and Pinelli-Saavedra, 2003). Moreover, cosmetic products such as skin care, lipstick and some kinds of soap contain vitamin E in order to prevent aging (Getoff, 2007) and increase the protection against irradiation (El-Nahas *et al.*, 1993).

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1.1.3. Dietary sources of vitamin E

According to the report of U.S. Department of Agriculture, the U.S. Recommended Daily Allowance (RDA), 15 milligrams, of α-tocopherol equivalents per day should be served for both men and women ages 19 and older. Therefore, vitamin E needs to be consumed by human. Table 2 shows good sources of vitamin E

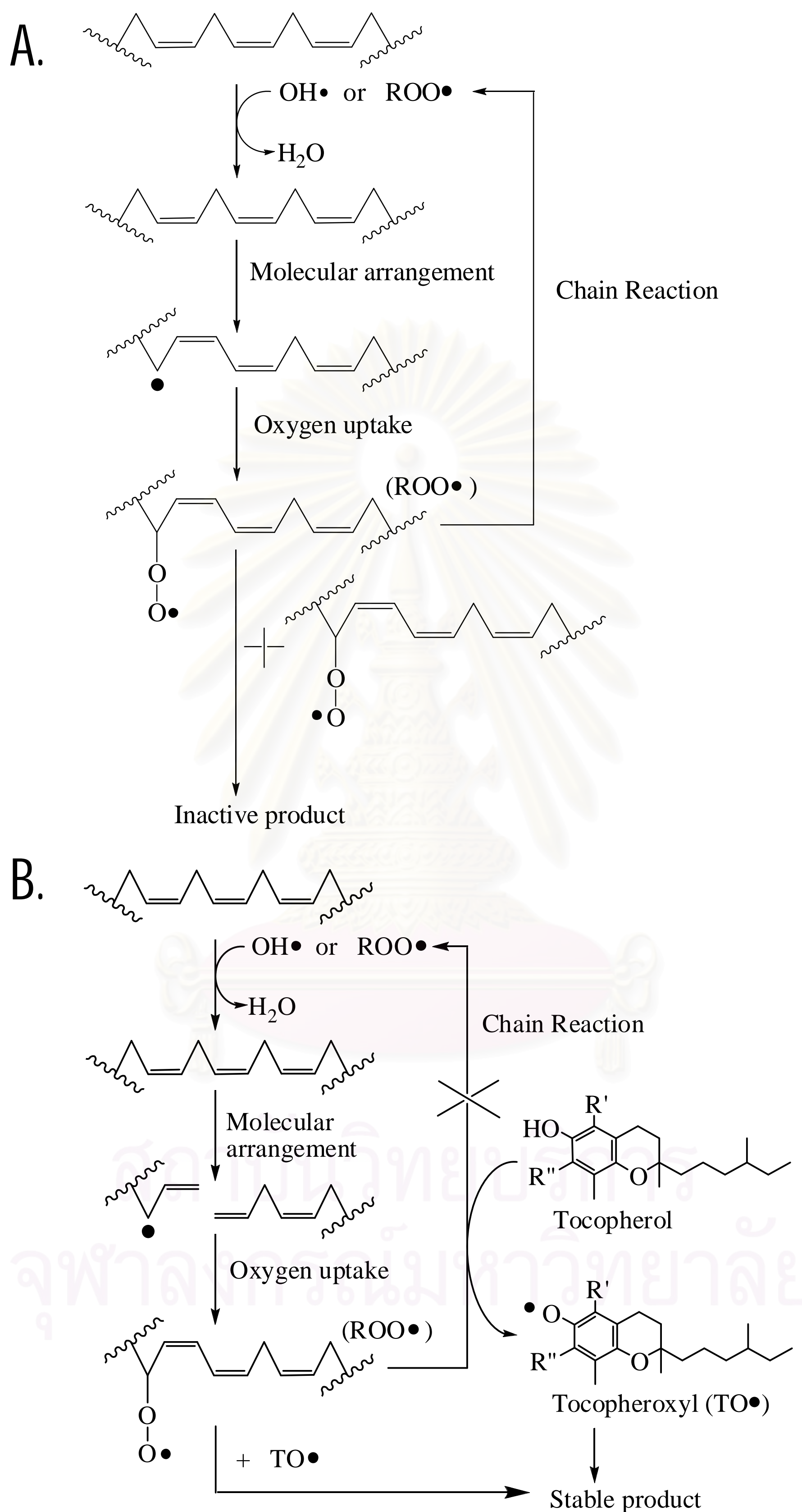

Figure 5. Schematic diagram showing basic sequence of Lipid peroxidation (Young and McEneny, 2001) (A) and inhibition of lipid peroxidation by α-tocopherol (Burton and Traber, 1990) (B).

Table 2 Good sources of vitamin E from uncooked source (Mosure, 2004)

Food	Serving size	Milligrams	%RDA
Egg, whole, fresh	1 large	0.88	5.8
Almond oil	1 tablespoon	5.3	35.3
Corn oil	1 tablespoon	1.9	12.6
Cottonseed oil	1 tablespoon	4.8	32.0
Olive oil	1 tablespoon	1.6	10.6
Palm oil	1 tablespoon	2.6	17.3
Peanut oil	1 tablespoon	1.6	10.6
Safflower oil	1 tablespoon	4.6	30.6
Soybean oil	1 tablespoon	1.5	10.0
Sunflower oil	1 tablespoon	6.1	40.6
Vegetable-oil spray	2.5 second spray	0.51	3.4
Wheat-germ oil	1 tablespoon	20.3	135.3
Tomato juice	6 fluid ounces	0.4	2.6
Apple with skin	1 medium	0.81	5.4
Mango, raw	1 medium	2.32	15.4
Macaroni pasta, enriched	1 cup	1.03	6.8
Spaghetti pasta, enriched	1 cup	1.03	6.8
Almonds, dried	1 ounce	6.72	44.8
Hazelnuts, dried	1 ounce	6.7	44.6
Peanuts, dried	1 ounce	2.56	17.0
Pistachio nuts, dried	1 ounce	1.46	9.7
Walnuts, English	1 ounce	0.73	4.8
Margarine (Mazola)	1 tablespoon	8.0	53.3
Avocado, raw	1 medium	2.32	15.4
Spinach, raw	½ cup	0.53	3.5
Tomato, red, raw	1 tomato	0.42	2.8

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including the value of %RDA. However, vitamin E can be lost during preparation, cooking or storage. The intake of it should be slightly more than the values indicated in Table 2 or food should be stored in airtight containers and avoid exposing them to light (Mosure, 2004).

1.1.4. Disadvantages of vitamin E and the improvement

Although, vitamin E has been applied for multipurpose uses at present, there are still some limitations. Not only is the solubility of vitamin E low, but its antioxidant activity is also not effective in some conditions. For example, being the most biologically active in eight homologues, α-tocopherol cannot prevent the oxidation of soybean phosphatidyl choline (PC) and rabbit erythrocytes from water-soluble radical initiator. In contrast, this situation cannot occur when the phytyl group on α-tocopherol is substituted with methyl group. This shows the problem of long phytyl side chain on α-tocopherol that makes no oxidative prevention in aqueous phase (Niki *et al.*, 1985 and 1988). Therefore, many researchers have been studying on how the antioxidant properties in aqueous solution can be improved by using various methods. For example, phosphatidyl and phosphate ester derivatives of vitamin E were synthesized via enzymatic process (Koga *et al.*, 1994 and Miyamoto *et al.*, 1998). The former derivative can be produced from hydroxyethyl derivative of α-tocopherol and egg yolk PC by transphosphatidylation catalyzed by phospholipase D from *Streptomyces lydicus*. Phosphatidyl derivative was less effective as antioxidant than α-tocopherol when unilameller egg yolk PC liposomes were exposed to either water-soluble or lipid-soluble radical initiator (Koga *et al.*, 1994). Hence, this derivative was treated by phospholipase C in order to hydrolyze phospholipid side chain bond that gave the phosphate ester derivative (Chromanol phosphate, Ch-P) of α-tocopherol (Figure 6). Ch-P was highly soluble in aqueous phase and showed higher antioxidant activity than α-tocopherol and phosphatidyl derivative in Fe (III) / ascorbic acid-catalyzed peroxidation of a fish oil emulsion and the autoxidation of a rat brain homogenate (Miyamoto *et al.*, 1998). In

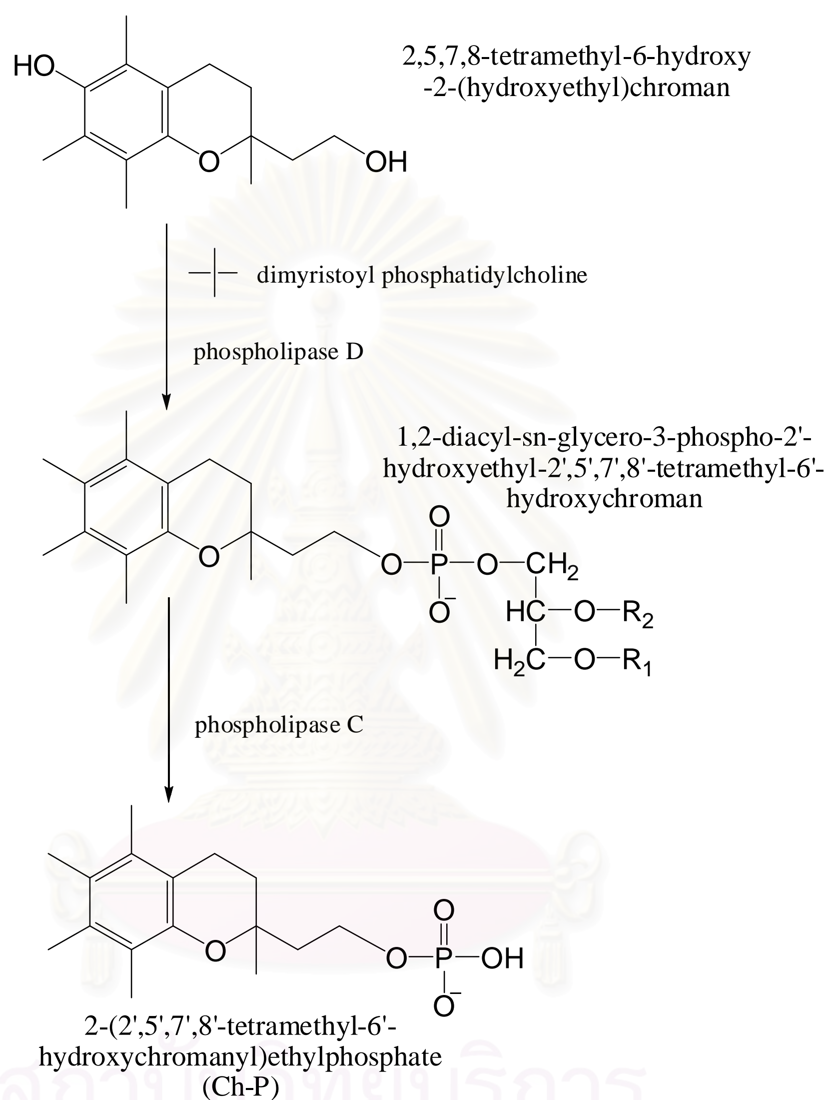

Figure 6. Ch-P (2-(2',5',7',8'-Tetramethyl-6'-hydroxychromanyl)ethylphosphate) synthesis (Niki *et al.*, 1985 and 1988).

general, most of vitamin E analogues, which are modified at 6-*O*-position, their antioxidant activity are often lost. However, the latest found derivative, vitamin E phosphate, showed the positive effect in antioxidant although its 6-*O* position was substituted with a phosphate moiety (Figure 7). It was not only found in nature but also synthesized by chemical method in the form of α-tocopherol phosphate disodium salt (TP sodium salt) which possibly was a reserved form of α-tocopherol in animal tissues (Gianello *et al.*, 2005 and Munteanu *et al.*, 2004). Its solubility in water was very low because it contained phytyl side chain like α-tocopherol.

1.2 Trolox

1.2.1 Structure and properties of Trolox

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, $C_{14}H_{18}O_4$), is phenolic antioxidant and partially water-soluble, the phytyl side chain of which is replaced by carboxylic moiety (Figure 8). Some properties of Trolox are shown in Table 3 (Calbiochem, 2003).

1.2.2. Applications of Trolox

At the present time, Trolox has been used as a model instead of α-tocopherol for the antioxidant study in aqueous solution resulting from the similarity in their structures. Therefore, Trolox is used as standard for TEAC (Trolox Equivalent Antioxidant Capacity) or DPPH (1,1-diphenyl-2-picrylhydrazyl) assay in order to measure antioxidant activity of some food products and medicine in aqueous solution. Examples include fruit juices, tea and plant extracts. This can be carried out by scavenging of long-lived radical anions or cations such as ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate)) or DPPH (Plumb *et al.*, 1999, Chen *et al.*, 2005 and Wong *et al.*, 2006). For TEAC assay, radicals are generated through the peroxidase activity of metmyoglobin in the presence of hydrogen peroxide.

Table 3 Physical and chemical properties of Trolox (Calbiochem, 2003)

Property	Characteristics
Name	6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
Formula	$C_{14}H_{18}O_4$
Molecular weight	250.3
Physical state and appearance	Off-white solid crystalline powder
Melting point	188 to 190 ^{O}C (370.4 to 374°F)
Refractive index	+0.32^{O} in ethanol, -3.0^{O} in benzene
Solubility	Partially soluble in cold water (0.5 mg/mL) Easily soluble in methanol (160 mg/mL)
Instability and Incompatibility	Stable Incompatible with oxidizing agents
Toxicity	Acute oral toxicity (LD_{50}): ~4300 mg/kg [Rat]

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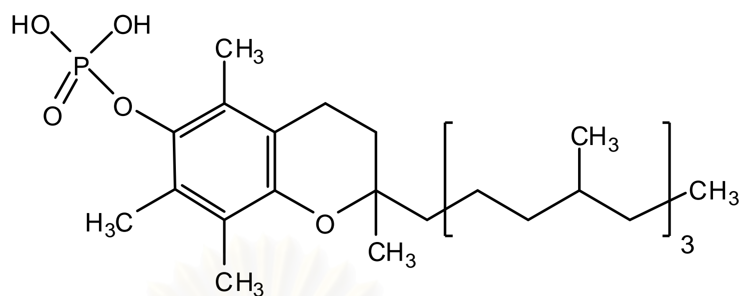

Figure 7. Structure of vitamin E phosphate (RRR-a-Tocopheryl phosphate) (Ogru *et al.*, 2003).

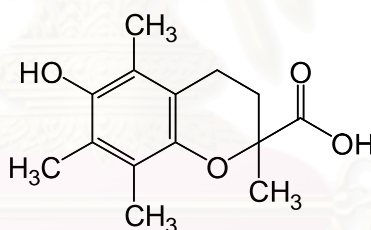

Figure 8. Structure of Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Murase *et al.*, 1997).

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Hence, radical is added by Trolox and detected spectrophotometrically the reduction of color at 734 nm (Figure 9A) (van den Berg, *et al.*, 1999). On the other hand, DPPH is a free radical itself and can react with any antioxidant in solution. DPPH radical can be detected by the reduction of color at 515 nm, after the reaction has finished (Figure 9B) (Brand-Willams, *et al.*, 1995).

1.2.3. Limitations of Trolox and glucoside derivative of Trolox

Although Trolox has a wide range of use, its solubility in water is low in practice (0.16 mg/mL). In 1997, Trolox was used as a substrate to synthesize the glucoside product (Murase *et al.*, 1997). Trolox was reduced to 2-hydroxyl-2,5,7,8-tetramethylchroman-6-ol (TM) by lithium aluminum hydride ($LiAlH_4$), and then TM was glucosylated by α-glucosidase to increase its solubility (Figure 10). The product, 2-(α-D-glucopyranosyl)methyl-2,5,7,8-tetramethylchroman-6-ol (TMG), showed approximately 6,000 times higher solubility than that of Trolox with the same degree of the scavenging activity against DPPH. Therefore, transglycosylation reaction is one of the appropriate methods for the improvement of solubility properties of Trolox without losing the antioxidant properties. The antioxidant activity of TMG was investigated to compare with that of α-tocopherol and ascorbic acid in the peroxyl radical scavenging activity against a 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) as a water-soluble radical generator and 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN) as a lipid-soluble radical generator in solution, liposomal suspension and human plasma. The effect of TMG on metal ion induced lipid peroxidation was also examined by using liposomal suspension with cupric metal ion and rat brain homogenate. TMG had the same scavenging activity as α-Tocopherol in the prevention of lipid peroxidation and ascorbic acid in the peroxidation in aqueous phase. Moreover, TMG had a higher antioxidant activity than ascorbic acid in the liposomal peroxidation and could inhibit the lipid peroxidation in the presence of transition metal ions (Murase *et al.*, 1998). TMG was also applied in the radioprotection because some flavonoids from plant, orientin and vicenin from leaves of Indian plant - *Ocimum sanctum*, have not only shown significant protection against

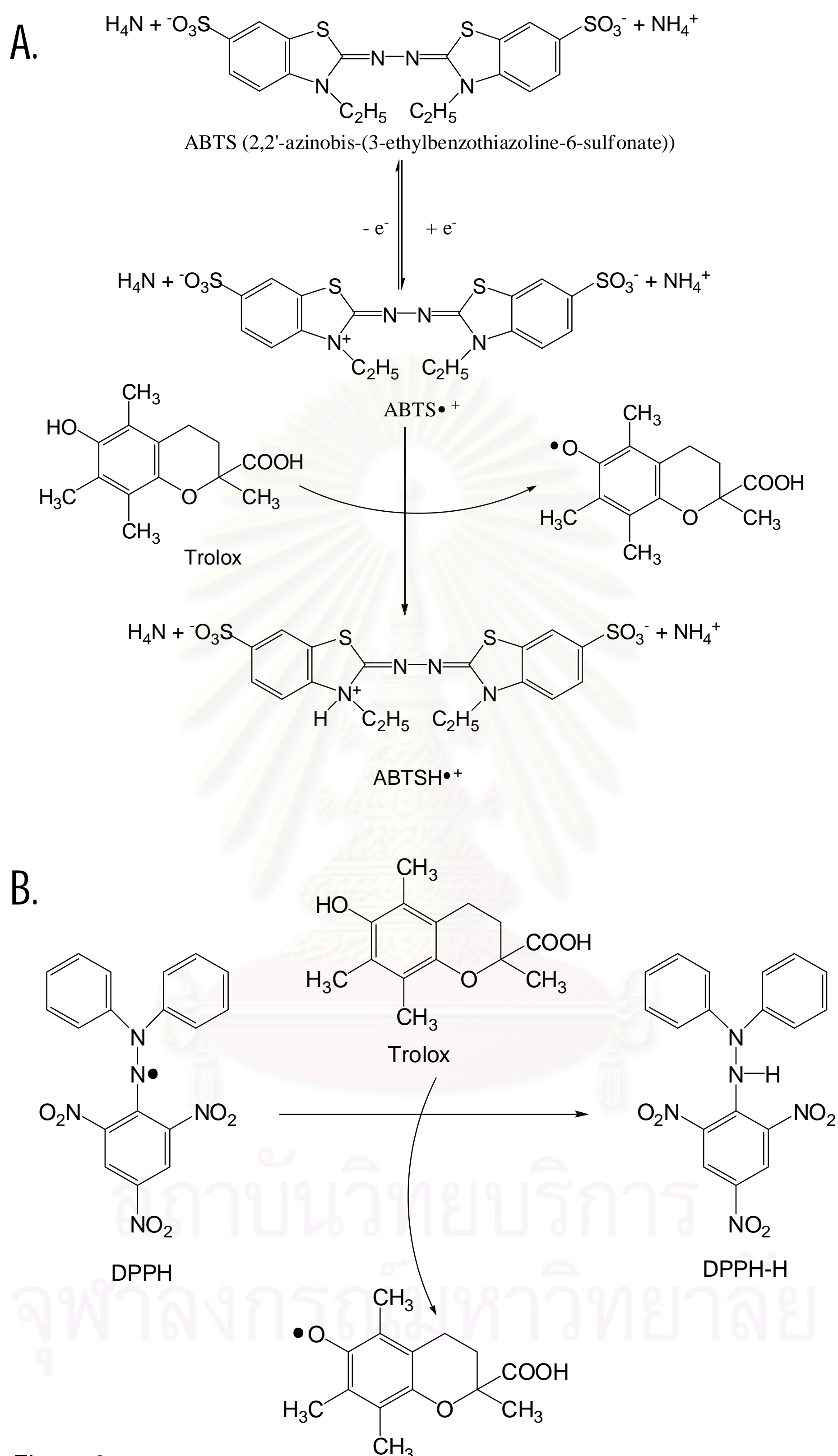

Figure 9. Schematic diagrams showing scavenging methods.

A.TEAC assay

B.DPPH assay

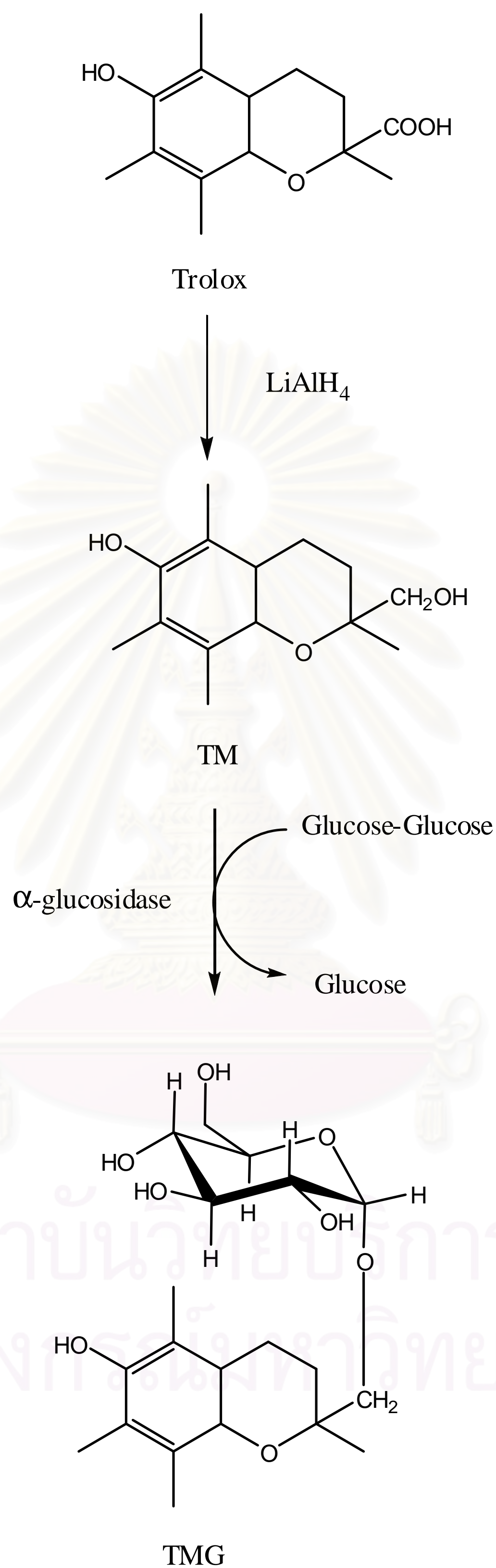

Figure 10. Transglucosylation to Trolox by α-glucosidase using maltose as glucosyl donor (Murase *et al.*, 1997).

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irradiation lethality and chromosomal aberrations in vivo, but also shown the effective level of their antioxidant activity against free radical hydrogen peroxide (Vrinda and Uma devi, 2001 and Nayak *et al.*, 2006). In the radioprotection experiment, mice were treated by TMG before and after gamma irradiation comparing with non-irradiated mice. Injection of TMG after whole body gamma-radiation significantly decreased the chromosomal aberration in bone marrow up to 60% (Satyamitra *et al.*, 2001). From these reports, TMG is the interesting compound that has advantages for therapeutic use and radioprotectant composition in cosmetics.

1.3 Transglycosylation

1.3.1 Transglycosylation by chemical methods

The chemical synthesis was chosen to produce many compounds for years. α-Tocopheryl oligosaccharide was synthesized by BF_3-etherate with acetylated saccharides as donor. Although the many glycoside products were produced, there are only three glycoside products were found to be soluble in water, α-tocopheryl β-maltotetraoside, α-tocopheryl β-maltohexaoside, α-tocopheryl β-maltoheptaoside (Lahmann and Thiem, 1997). Two neosaponins was synthesized by BF3-etherate using diosgenin as an acceptor and mimosatetraose and fabatriose as donors. These neosaponins at the concentration of 0.5 mg/mL had a thirty percent and fifty-eight percent hepatoprotection in rat (Ikeda *et al*, 1998). From these reports, it can be seen that chemical transglycosylation can synthesize many glycosyl derivatives from initial acceptor. However, the method is complicated to apply for industry especially the production of higher glycosyl moiety. Therefore, the enzymatic synthesis is used for complement or substitution to the chemical one.

1.3.2 Transglucosylation by α-glucosidase

α-glucosidase (α-1.4-glucosidase, α-D-glucosidase, maltase) is the hydrolase enzyme group (EC 3.2.1.20) that rapidly hydrolyzes the terminal, nonreducing 1,4-α-

glucosidic linkages of oligosaccharides with the release of α-D-glucose, relative to polysaccharides, which are hydrolyzed relatively slowly, or not at all (IUBMB, 2006). Moreover, α-glucosidase can catalyze transglucosylation to synthesize α-glucosylated compounds by using the simple glycosyl donors. This contradicts with glycosyltransferases, which use the complex glycosyl donors in transglycosylation (Crout and Vic, 1998). The solubility of some compounds in water can be increased when glucose moiety was introduced into the molecules.

α-Arbutin is one of the skin-whitening agents and is widely used as components in cosmetics industry. Its action is the inhibition of the production of melanin (Funayama *et al.*, 1995). It could be synthesized by α-glucosidase from *Saccharomyces cerevisiae* using hydroquinone and maltose as a glucosyl donor. The yield of this reaction was 4.6% molar yield with respect to hydroquinone (Prodanovic *et al.*, 2005). 2-Deoxy sugars are frequenly found in nature as the bioactive oligosaccharides and they can be used as antibiotics, antitumor drugs, and antiparasitic agents. Ethyl 2-deoxy-α-D-*arabino*-hexopyranosyl-(1,4)-β-D-thioglucopyranoside and ethyl 2-deoxy-α-D-*arabino*-hexopyranosyl-(1,6)-β-D-thioglucopyranoside were synthesized by α-glucosidase from *Aspergillus niger* using *p*-nitrophenyl α-D-glucopyranoside (Glcα-*O*-*p*NP) and ethyl β-D-thioglucopyranoside (Glcβ-*S*-Et) as a glycosyl donor and acceptor, respectively. The isolated yield of ethyl 2-deoxy-α-D-*arabino*-hexopyranosyl-(1,4)-β-D-thioglucopyranoside and ethyl 2-deoxy-α-D-*arabino*-hexopyranosyl-(1,6)-β-D-thioglucopyranoside based on sugar donor were 6.72% and 46.6%, respectively. L-Menthol, one of the popular flavones for food, medicines and cosmetics flavoring, was added with glucose by two-step enzymatic reaction system: α-glucosidase and cyclodextrin glycosyltransferase (CGTase). Solubility in water of *l*-menthyl α-maltosides, end product of this reaction, is expanded 1570 times more than *l*-menthyl α-glucosides, the product from the first step (Do *et al.*, 2002).

1.3.3 Transglycosylation by cyclodextrin glycosyltransferase

Cyclodextrin glycosyltransferase (CGTase; 1→4-α-D-glucopyranosyltransferase or cyclomaltodextrin glucanotransferase) is a starch degrading enzyme relating to

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α-amylase family. CGTase belongs to the transferase group of enzymes (EC 2.4.1.19) which are produced by various microorganisms especially *Bacillus* species. However, there are also reports on CGTase production in different kinds of bacteria. Some of the bacteria that can produce CGTase are listed in Table 4.

CGTase is an extracellular enzyme which can catalyze transglycosylation reaction in 3 forms. CGTase can catalyze cyclization (conversion of starch to cyclodextrins - CDs are cyclic and non-reducing oligosaccharides which are mainly found in 3 forms: α-, β- and γ-cyclodextrin that are built up from six, seven and eight D-glucopyranosyl units, respectively as shown in Figure 11 (Bender, 1986 and Szetjli, 2004). Moreover, this enzyme also catalyzes a coupling reaction (cleavage of α-glycosidic bond of CD rings and transfer glycosyl group of linear maltooligosaccharides to some types of acceptors) and a disproportionation reaction (transfer glycosyl group from linear maltooligosaccharides to some types of acceptors) by ping-pong mechanism. The proposed models of the events taking place in the CGTase-catalyzed are shown in Figure 12. A scheme of CGTase-catalyzed transglycosylation reactions (van der Veen, 2000) is shown in Figure 13.

The efficiency of each mechanism depends on the number of glucopyranosyl residue in reaction mixture. In other words the cyclization takes place most effectively when substrates have glucopyranosyl units from 16 to 80 molecules. Meanwhile, the coupling and disproportionation reaction have rapidly occurred in reaction mixture which has 5-14 units and more than 100 units of glucopyranose respectively. Nevertheless, the disproportionation reaction can still poorly occur in reaction mixture with 5-14 units of glucopyranose (Szejtli, 1988). In general, the application of CGTase is CD production by cyclization reaction. However, CGTase can be used for synthesizing derivatives of some compounds so as to improve their disadvantageous properties via disproportionation reaction. 2-*O*-α-D-Glucopyranosyl-*L*-ascorbic acid (2G-AA) was a classical example of CGTase transglycosylation study that used ascorbic acid and α-cyclodextrin as an acceptor and donor, respectively (Tanaka *et al.*, 1991). Not only α-CD but also most of saccharides such as cyclodextrin, dextrin and starch can be used as donor except monosaccharide (Jun *et al.*, 2001).

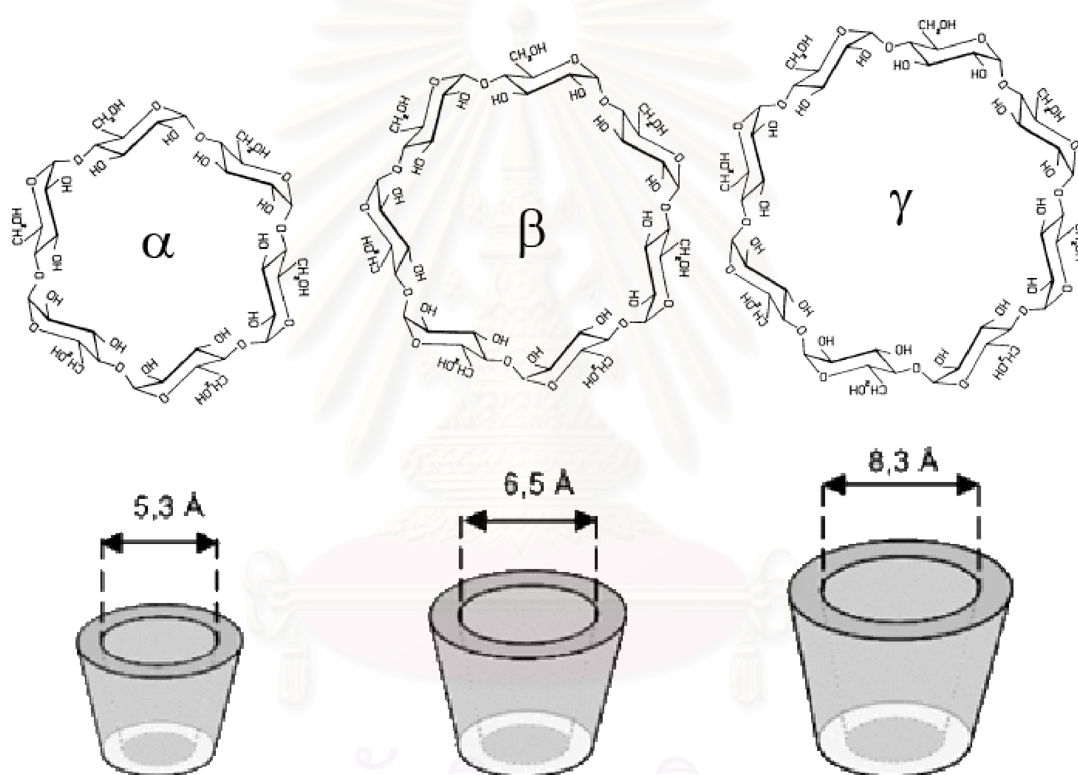

Figure 11. Natural CD – α-cyclodextrin, β-cyclodextrin and γ-cyclodextrin (Szetjli, 2004).

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Table 4 Properties of CGTase from some microorganisms

Organism	Main Product	Optimum pH	Optimum Temperature (^{o}C)	MW (kDal)	References
Paenibacillus Pabuli US132	β-CD	6.5	65	70	Jemli *et al*., 2007
Bacillus agaradhaerens LS-3C	β-CD	9.0	55	110	Martins *et al*., 2002
Anaerobranca gottschalkii	β-CD	5.0-10.0	55-70	78	Thiemann *et al*., 2004
Bacillus sp. 7-12	β-CD, γ-CD	8.5	60	69	Cao *et al*., 2004
Bacillus sp. G1	β-CD	6.0	60	79	Sian *et al*., 2005
Paenibacillus sp. RB01	α-CD, β-CD	7.0	70	45	Yenpetch, 2002
Thermococcus sp.B1001	α-CD	7.0	85	83	Tachibana *et al*., 1999
Bacillus circulans A11					
Isoform 1	β-CD	6.0-7.0	40	72	Kaskangam, 1998
Isoform 2	β-CD	6.0-7.0	40	72	
Isoform 3	β-CD	6.0	50	72	
Isoform 4	β-CD	7.0	50-60	72	
Paenibacillus sp. F8	β-CD	7.5	50	72	Larsen *et al*., 1998

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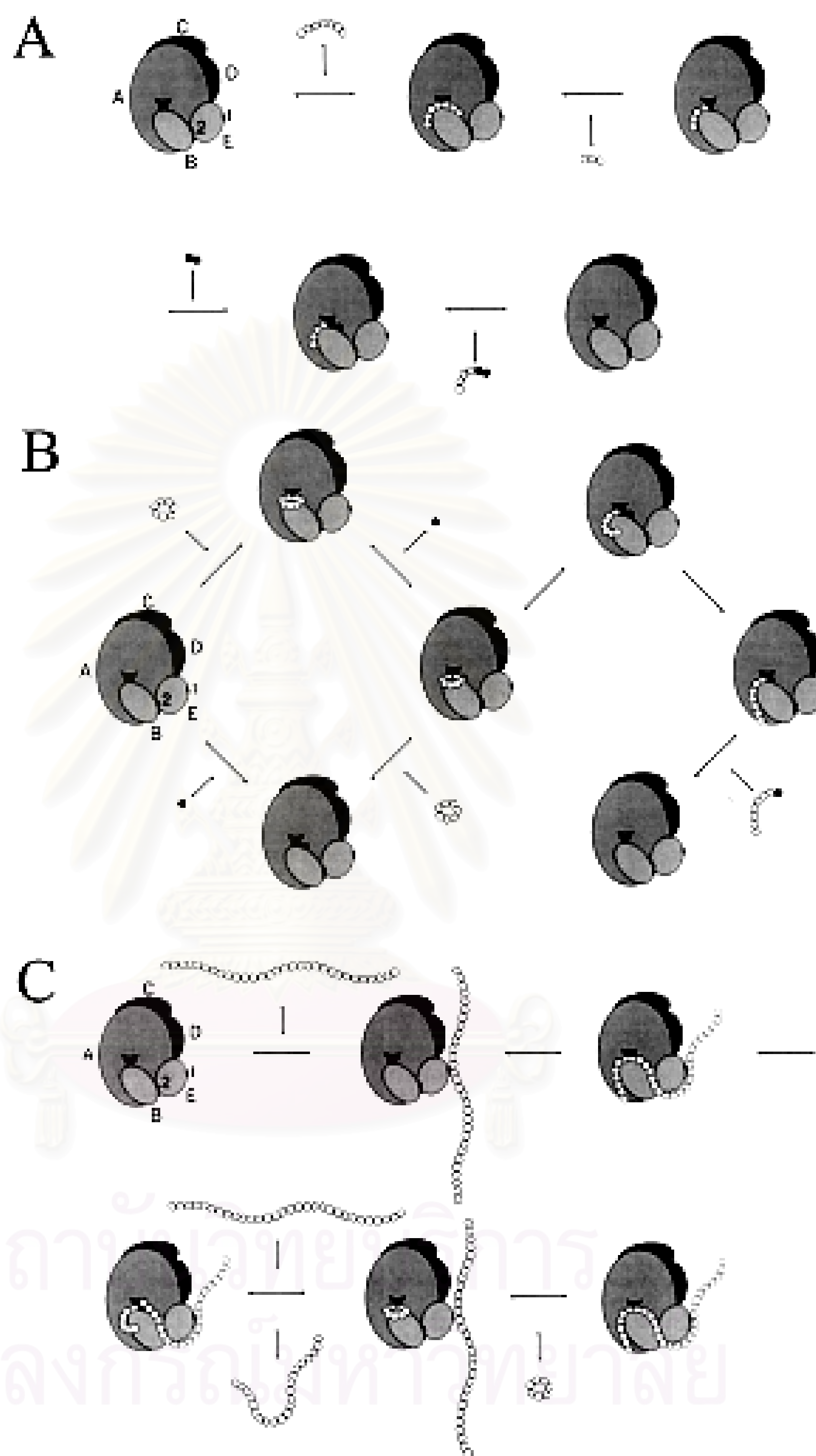

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Figure 12. Proposed models of the events taking place in the CGTase-catalyzed reactions. (van der Veen *et al.*, 2000) (A) Disproportionation, (B) coupling and (C) cyclization. The different CGTase domains are indicated (A, B, C, D and E). 1 and 2 indicate the maltose binding sites on the E-domain. The triangle indicates the cleavage site in the active site. Circles represent glucose residue; acceptor residues are represented in black.

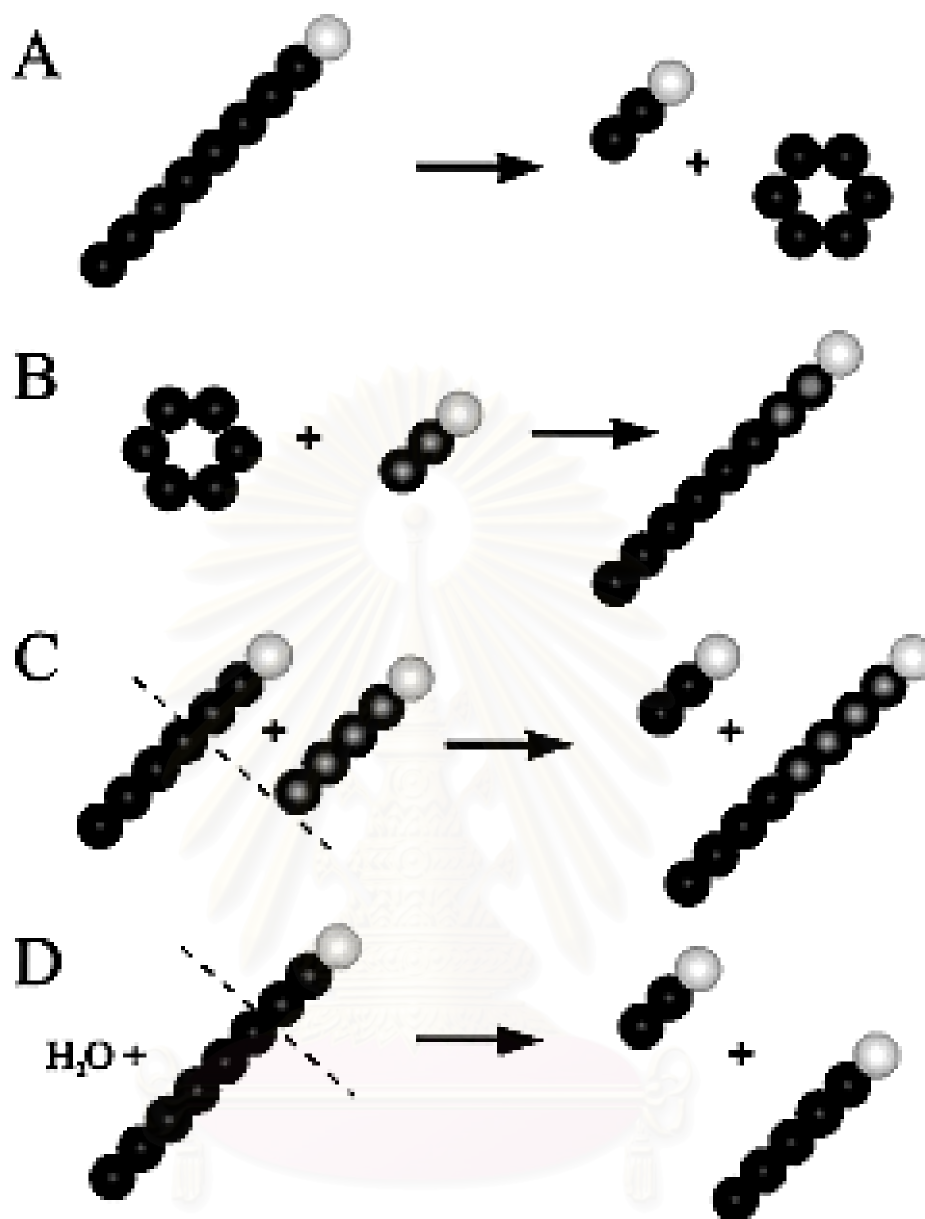

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Figure 13. Schematic representations of the CGTase-catalyzed transglycosylation reactions. (A) Cyclization; (B) coupling; (C) disproportionation and (D) hydrolysis. The shaded circles represent glucose residues; the white circles indicate the reducing end glucoses.

At present, 2G-AA is utilized as a substrate to synthesize 6-*O*-acyl-2G-AA that is used as components in cosmetics with high stability and active in vivo and can probably be used as food supplements in the future (Tai *et al.*, 2002). *O*-α-D-glucosyl glycerol (Glc-GL) was synthesized by CGTase using glycerol and soluble starch as an acceptor and donor, respectively (Nakano *et al.*, 2003). Glc-GL has been found in Japanese traditional food such as miso, mirin and sake. In addition, Glc-GL shows the sign of healthy food ingredients. Because Glc-GL exhibits about half the sweetness of sucrose, high-thermal stablility, low Maillard reacitivity, non-carcinogenicity, and low digestibility by rat intestinal enzymes, it is expected to be low calories ingredients in food and beverages industry (Takenaka *et al.*, 2001). Maltooligosyl-fructofuranosides, which is a non-cariogenic sweetener, could be produced by immobilized CGTase using starch as donor and sucrose as acceptor (Martin *et al.*, 2004). Glucosyl-xylitol, the functional alternative sweetener with stimulation effect on the growth of bifidobacterium, was synthesized by CGTase using xylitol as an acceptor and extruded starch as a donor (Kim *et al.*, 1998). 3^G-α-D-glucopyranosyl neohesperidin was also synthesized using transglycosylation activity of CGTase to reduce bitterness of neohesperidin – one of the natural flavonoids in lime and lemon juice. This neohesperidin glycoside was approximately 1500 times more soluble in water than neohesperidin and 10 times less bitter (Kometani *et al.*, 1996).

The starch and cyclodextrin research unit at the Department of Biochemistry, Faculty of Science, Chulalongkorn University has been working on CGTase isolated from thermotolerant *Paenibacillus* sp. RB01. This strain had previously been screened from a hot spring soil area in Ratchaburi and CGTase from this strain was first partially purified and characterized by Tesana (2001). Then, this enzyme was completely purified and biochemically characterized by Yenpetch (2002). The temperature condition for RB01 growing was in the range of 30-45°C, while the optimized condition for CGTase production was 40°C. The optimized pH and temperature for dextrinizing activity are 7-9 and 45-55°C, respectively (Yenpetch, 2002). Since, the advantages of CGTase from this strain of bacteria is thermotolerant property, it is the appropriate enzyme to apply for the industry. There has been an increasing interest in developing scalable synthetic or

enzymatic routes in order to modify various physiologically active substances. CGTase has recently been widely used for the modification and development of new functional properties of various substances. The aim of this research is a two-step reaction sequence by α-glucosidase from *Saccharomyces cerevisiae* (Murase *et al.*, 1997) and CGTase from *Paenibacillus* sp. RB01 for the production of a novel vitamin E glycoside. Optimization of the production condition will also be investigated. Then, the glycoside product will be purified and structurally elucidated. Finally, the large-scale production of this product will be performed so as to apply this method for industrial purpose. Since TMG could be applied in therapeutic use such as a radioprotector and a drug for prevention from gastric mucosal injury by aspirin (Nair *et al.*, 2005 and Isozaki *et al.*, 2005), the novel obtainable vitamin E glycoside from this method was expected to have higher solubility than that of TMG with the same antioxidant activity as the substrate, Trolox. Thus, it can also be further used as the water-soluble antioxidant of clinical use for the diseases induced by oxidative damage.

The objectives of this research were:

I) To study the transglycosylation to vitamin E with α-glucosidase from *Saccharomyces cerevisiae* and CGTase from *Paenibacillus* sp. RB01

II) To determine the optimum conditions for the production of the vitamin E glycoside

III) To purify and elucidate the structure of the vitamin E glycoside product

IV) To evaluate its use by testing its solubility in water and free-radical scavenging activity

CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Analytical column for HPLC: Inertsil® ODS-3 4.6x250 mm I.D. 5 μm, GL Science Inc., Japan

Analytical balance: AB204-S, Mettler-Toledo, Switzerland

Autoclave: MLS-3020, Sanyo electric Co., Ltd., Japan.

Autopipette: Nichipet EX, Nichiryo, Japan.

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

Centrifuge, microcentrifuge, high speed centrifuge: MIKRO 22R, Hettich Laborapparate, Germany

Electrophoresis unit: Mini-PROTEAN® 3 Electrophoresis, Bio-Rad, U.S.A.

Freeze-dryer: LYPH-LOCK, LABCONCO, U.S.A.

High Performance Liquid Chromatography: Hewlett-Packard series 1050, Japan

Incubator: Haraeus, Germany

Incubator shaker: Psyco-Therm, New Brunswick Scientific Co., Ltd., U.S.A.

Laminar flow: Model BVT-124, International Sciencetific Supply Co., U.S.A.

Magnetic stirrer and heater: Model 512p-2, Barnstead/Thermolyne Corporation, U.S.A

Mass spectrometry: MicroTOF, Bruker Daltonics Inc., U.S.A.

Nuclear Magnetic Resonance Spectrometer: Varian Gemini 400 MHz, Varian, U.S.A.

pH Meter: pH900, Precisa, Switzerland

Precision balance: PB303-S, Mettler-Toledo, Switzerland.

Rotavapor R-200, BUCHI, Switzerland.

Heating bath B-490, BUCHI, Switzerland.

Recirculating chiller B-740, BUCHI, Switzerland.

UV-VIS Spectrophotometer: DU650 Spectrophotometer, Beckman, USA

VIS Spectrophotometer: 6400 Spectrophotometer Jenway, LABQUIP, England

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

Water bath: Memmert, Germany

2.2 Chemicals

acetic acid 100%: BDH, England

acetonitrile (HPLC grade): Labscan, Thailand

amberlite XAD-4: Fluka, Switzerland

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amyloglucosidase (Glucoamylase) from *Aspergillus niger* 70.7 U/mg: Fluka, Switzerland

α-glucosidase (maltase) 5.8 U/mg: Fluka, Switzerland

bacto-peptone: Difco Laboratories, U.S.A.

bacto-agar: Britania, Argentina

beef Extract: Difco Laboratories, U.S.A.

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

β-cyclodextrin: Nihon shokukin kako company Ltd., Japan

calcium chloride: Merck, Germany

comassie brilliant blue G-250: Sigma, U.S.A.

comassie brilliant blue R-250: Sigma, U.S.A.

cornstarch: Maizena, Thailand

dialysis tubing: Sigma, U.S.A.

dimethyl sulfoxide (synthesis grade): Merck, Germany

dipotassium hydrogen phosphate (K_2HPO_4): Univar, Australia

ethanol absolute: Carlo Erba, France

ethylenediaminetetraacetic acid (EDTA), Scharlau, Spain

ethyl acetate: Scharlau, Spain

36.5-38.0% hydrochloric acid: J.T.Baker, U.S.A.

iodine: Fluka, Switzerland

lithium aluminum hydride ($LiAlH_4$): Fluka, Switzerland

magnesium heptahydrate ($MgSO_4.7H_2O$): Univar, Australia

maltose: Conda, Spain

2-mercaptoethanol: Scharlau, Spain

methanol: Merck, Germany

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

85% orthophosphoric acid: Univar, Australia

peptone from meat: Merck, Germany

phenolphthalein: BDH, England

potassium dihydrogen phosphate (KH_2PO_4): Scharlau, Spain

potassium Iodide: Univar, Australia

TLC Slilica gel 60 plate F254, Merck, Germany

silica gel 60 for column chromatography: Merck, Germany

sodium acetate: BDH, England

sodium carbonate: BDH, England

sodium chloride: BDH, England

sodium hydroxide: BDH, England

soluble starch, potato: Scharlau, Spain

standard molecular weight marker protein: Sigma, U.S.A.

sulfuric acid: BDH, England

tetrahydrofuran (THF), Labscan, Thailand

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

tris: USB corporation, U.S.A.

trolox, Fluka, Switzerland

yeast extract: Scharlau, Spain

2.3 Bacteria

Paenibacillus RB01 was isolated from hot spring soil in Rachaburi province, Thailand and screened for CGTase activity by Tesana, 2001.

2.4 Media preparation

2.4.1 Medium I

Liquid medium I, composed of 0.5% (w/v) beef extract, 1.0% (w/v) peptone from meat, 0.2% (w/v) NaCl, 0.2% (w/v) yeast extract and 1.0% (w/v) soluble starch, was prepared and adjust to pH 7.2. In the case of solid medium, 1.5% (w/v) bacto-agar was added. Medium I was sterilized by autoclaving at 121°C for 15 minutes.

2.4.2 Horikoshi medium (HK)

HK was prepared to produce CGTase. Medium formula is slightly modified from Horikoshi, 1971, consisted of 1.0% (w/v) peptone from meat, 0.5% (w/v) yeast extract, 1.0% (w/v) soluble starch, 0.1% (w/v) K_2HPO_4, 0.02% (w/v) $MgSO_4.7H_2O$ and 0.75% (w/v) Na_2CO_3 with the pH of 10.1-10.2. Medium was sterilized as described above.

2.5 Cultivation of bacteria

2.5.1 Starter cultivation

A colony of *Paenibacillus* RB01 was grown in 50 mL of starter Medium I in 250 mL conical flask at 37 ^{O}C with 250 rpm incubator shaking until A_{660} measurement by spectrophotometer reached 0.3-0.5 unit.

2.5.2 Enzyme production

Starter *Paenibacillus* RB01 (1.0 % (v/v)) was transferred to HK broth in a 1,000 mL Erlenmeyer flask at 40 ^{O}C with 250 rpm incubator shaking. Starter was cultured for

60 hours then harvested and cells were removed by refrigerated centrifugation at 3,000 x g for 30 minutes. Crude CGTase was collected as supernatant and kept at 4 ^{O}C for purification.

2.6 Partial purification of CGTase

CGTase was partially purified by starch adsorption method with slightly modification from Kato and Horikoshi, 1984

Corn starch was dried at 100 ^{O}C for 2-3 hours, and was cooled to room temperature. It was gradually sprinkled into crude CGTase from the previous step to 5% (w/v) concentration. The mixture was 3-hour-continuous stirred at 4 ^{O}C and then starch cake was recovered from 4 ^{O}C centrifugation at 2,390 x g for 30 minutes. The starch cake was washed twice with 10 mM Tris-HCl containing 10 mM $CaCl_2$ pH 8.5 (TB1) and centrifuged at 4,039 x g 4 ^{O}C for 30 minutes. The washed starch cake was kept in order to elute CGTase with TB1 containing 0.2 M maltose pH 8.5 by three times of stirring at 4 ^{O}C for 30 minutes and 4 ^{O}C centrifugation at 7,745 x g for 30 minutes. The supernatant was collected, concentrated with viva flow and dialyzed twice by dialysis buffer (50 mM acetate buffer containing 5 mM $CaCl_2$ pH 6.0) at 4 ^{O}C.

2.7 Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE)

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

2.8 Enzyme assay

2.8.1 Dextrinizing activity assay

This method, which used iodine as indicator, measured the amount of starch digestion by CGTase. Enzyme sample (10 μl) was incubated with 0.3 mL of 0.2% (w/v) soluble starch in 0.2 M phosphate buffer pH 6.0 at 40°C for 10 minutes. The reaction was stopped by the addition of 4 mL of 0.2 M HCl, colored by adding 0.5 mL of 0.02% (w/v) I_2 containing 0.2% (w/v) KI and diluted by 5.2 mL of distilled water for A_{600} measurement. One unit of enzyme was defined as the amount of enzyme which produces 10% blue-color intensity reduction of the starch-iodine complex per minute under the described condition.

2.8.2 Cyclization activity assay

This experiment was used to measure β-CD formation from CGTase by using phenolphthalein as indicator. Enzyme sample (0.25 mL) was incubated with 1.25 mL of 6% (w/v) soluble starch at 70 ^{O}C for 30 minutes. Then the reaction mixture was heated for 5 minutes to stop the reaction. Oh point five milliliters of aliquot was taken to be mixed with 2 mL phenolphthalein solution and left at room temperature for 15 minutes. Its absorbance at 550 nm was measured by spectrophotometer. The amount of β-CD formation by the sample was calculated from the standard curve of β-CD concentration. One unit of enzyme was defined as the amount of 1 μmol of β-CD per minute under the above condition.

2.9 Protein determination

Bradford's protein determination (1976) was chosen to determine the amount of protein by using bovine serum albumin as standard. Enzyme sample (100 mL) was mixed with 0.1 mL of 1 M NaOH and 5 mL of protein reagent. The absorbance of the mixture was recorded at 595 nm after it was left for 5 minutes. One liter of protein

reagent is composed of 100 mg Coomassie blue G-250, 50 mL of 95% ethanol, 10 mL of 85% H_3PO_4 and distilled water

2.10 2-Hydroxyl-2,5,7,8-tetramethylchroman-6-ol (TM) preparation

This method is slightly modified from Murase *et al.* (1997). Trolox 1.0 g dissolved in distilled anhydrous tetrahydrofuran (THF) 30 mL and 0.5 g of lithium aluminum hydride ($LiAlH_4$) was added to a round bottom flask in an ice bath. The mixture was refluxed for 20 hrs. The reaction was stopped by concentrated HCl in an ice bath and solvent was evaporated at 40 ^{O}C by a rotary evaporator. TM was extracted by diethyl ether and diethyl ether was separated out at 40 ^{O}C by evaporation. Crude extract was purified by silica-gel column chromatography using hexane:ethyl acetate (7:3, v/v) as the solvent. The fraction was monitored by TLC (thin-layer chromatography) with the same solvent system. The TM fraction showed a single spot at R_f = 0.30 on TLC plate after the plate was dipped in $KMNO_4$ solution (See Appendix 2 for the composition of this solution). TM fractions were combined and solvent was evaporated. Pure (*R*,*S*)-TM was obtained as a white powder (0.8760 g yield). TM was confirmed by ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR dimethyl sulfoxide (DMSO) analysis to compare with the Murase *et al.* report (1997).

2.11 Transglycosylation reaction

2.11.1 Transglycosylation reaction to TM with α-glucosidase following the Murase *et al.* method (1997)

A reaction mixture (1.2 mL) was composed of 200 μl of 5% (w/v) TM in DMSO mixed well with 871 μl of 40% (w/v) maltose in 50 mM potassium phosphate buffer (pH 6.0) and 129 μl of α-glucosidase (30 U) in 50 mM potassium phosphate buffer (pH 6.5). The reaction mixture was incubated at 40 ^{O}C for 20 hours. Three hundred microliters of aliquot was withdrawn and analyzed by TLC and HPLC (high-performance liquid chromatography).

2.11.2 Transglycosylation reaction to Tocopherol monoglucoside (TMG) with CGTase

For total volume of 1 mL, Three hundred microliters of the reaction mixture was mixed well with 642 μl of 1.5 % (w/v) β-cyclodextrin in 50 mM potassium phosphate buffer (pH 6.0) and 58 μl CGTase (100 U). The reaction mixture was incubated at 40°C for 16 hours. After stopping reaction by 5-minute-heating, a 300 μl aliquot was withdrawn and analyzed by TLC and HPLC. Another 300 μl was checked by glucoamylase from *Aspergillus niger* (20 U) to investigate the transglycosylation products. The reaction mixture was incubated at 40°C for 20 hours and analyzed by HPLC.

2.12 The tocopherol glycoside (TMG_n) product determination

2.12.1 Thin layer chromatography (TLC)

TLC analysis was carried out on silica 60 coated on an aluminum plate using chloroform/methanol/acetic acid/water (40:10:2:1, by vol.) as a solvent system. TM and the glycoside products were detected by spraying with H_2SO_4/methanol (1:3 v/v) followed by charring.

2.12.2 High performance liquid chromatography (HPLC)

Reversed-phase HPLC analysis was performed using Hewlett-Packard 1050 series with ultraviolet (UV) detector. The reaction mixture was analyzed by Inertsil ODS-3 column (4.6 x 250 mm.; GL Science Inc., Tokyo, Japan) under the following condition: mobile phase, acetonitrile:water (40:60, v/v); flow rate, 0.5 mL/min; temperature 40°C. The eluent was monitored by an absorbance at 280 nm.

2.13 Optimization of TMG_n production

2.13.1 Optimization of temperature

The effect of temperature on the glycoside product synthesis was determined by using 300 μl of reaction mixture from transglycosylation to TM in section 2.11.1 mixed well with 1.5% (w/v) β-cyclodextrin in 50 mM potassium phosphate buffer (pH 6.0) and 58 μl CGTase (100 U). The selected temperatures for transglycosylation were 30, 40,

50, 60 and 70°C with 16 hours incubation. Then, the reaction was stopped by boiling for 5 minutes and analyzed by HPLC as mentioned in section 2.12.2

2.13.2 Optimization of pH

The appropriate pH for synthesis was determined by using a 300 μl of reaction mixture from transglycosylation to TM in section 2.11.1 mixed well with 1.5% (w/v) β-cyclodextrin in buffer solution and 58 μl CGTase (100 U). The fifty-millimolar buffers with pH ranging from 4.0-10.0 were used composing of acetate buffer (pH 4.0, 5.0 and 6.0), phosphate buffer (pH 6.0, 7.0 and 8.0) and Tris-glycine buffer (pH 8.0, 9.0 and 10.0) (Appendix 3). The optimum temperature determined earlier was used and the reaction was performed with 16-hour-incubation. Then, the reaction was stopped and analyzed by HPLC as described above.

2.13.3 Optimization of time

Time course of production was established by using a 300 μl of reaction mixture from transglycosylation to TM in section 2.11.1 mixed well with 1.5% β-cyclodextrin in optimum buffer solution with optimum pH from section 2.13.2 and 58 μl CGTase (100 U). The optimum temperature from section 2.13.1 was carried out with 0-48 hours incubation. After the incubation, the reaction mixture was analyzed by HPLC with condition as described in section 2.12.2.

2.13.4 Optimization of enzyme concentration

The purified TMG (1%, w/v), which obtained from the purification method in section 2.15.2, was dissolved in optimum buffer solution with optimum pH from section 2.13.2 mixed with 1.5% β-cyclodextrin in the same buffer solution. The mixture was incubated with 0-150 unit of CGTase at the optimum temperature from section 2.13.1 and optimum time from section 2.13.3. The HPLC was used to analyze the reaction mixture in the same condition as described in section 2.12.2.

2.13.5 Optimization of substrate concentration

A total of thirteen ratios between purified TMG and β-cyclodextrin were used for this test. The 1:0, 1:1, 1:1.5, 1:1.8, 1:2, 1:2.5 and 1:5 ratios in β-cyclodextrin variation, and 0:1, 0.25:1, 0.5:1, 0.75:1, 1.25:1 and 1.5:1 ratios in TMG variation were dissolved in optimum buffer solution with optimum pH from section 2.13.2 and mixed with CGTase at optimum unit from section 2.13.4. The optimum temperature and time for incubation were results from section 2.13.1 and 2.13.3, respectively. Then, the analysis of the reaction mixture by HPLC as mentioned in section 2.12.2 was used.

2.14 Yield of transglycosylation to TMG with CGTase

Then, all the appropriate conditions for vitamin E glycoside synthesis determined from section 2.13 were used to measure the efficiency of optimized reaction. Percent yield of product was determined from HPLC peak area and numerically calculated as:

$$\text{Transglycosylation yield (\%)} = \frac{\text{Peak area of the glycoside product}}{\text{Peak area of the TMG substrate at time} = 0} \times 100$$

2.15 Large scale production of TMG_n

2.15.1 Purification of TMG

Large amount of TMG production was performed in water bath. Ten milliliters of 5% (w/v) TM in DMSO was mixed with 43.55 mL of 40% (w/v) maltose in 50 mM potassium phosphate buffer (pH 6.0) and 6.45 mL of α-glucosidase (1,500 U) in 50 mM potassium phosphate buffer (pH 6.5). The reaction mixture was incubated at 40°C for 20 hrs and the reaction was stopped by boiling the reaction mixture for 5 minutes. Then, TMG was purified by passing through Amberlite XAD-4 resin which can eliminate DMSO from the mixture. Amberlite column (1 cm x 40 cm) was equilibrated with 30% methanol to wash DMSO and sugars out. TM and TMG were eluted with 80% methanol. DMSO and sugars were detected by TLC following the 2.12.1 section. TM and TMG were observed by spectrophotometer at 280 nm and identified by HPLC following the 2.12.2

section. All TMG fractions were combined. Solvent was removed by rotary evaporator followed by freeze-dryer.

2.15.3 Scale-up for TMG_n production

TMG and β-CD were dissolved in 50 mM potassium phosphate buffer (pH 6.0) at the optimized ratio obtained from section 2.13.5. The appropriate conditions which composed of results from section 2.13 were used for TMG_2 synthesis.

2.15.4 TMG_n purification

Product from section 2.15.3 was purified by silica gel column chromatography using ethyl acetate/methanol (6:1) as solvent. TMG and its product were observed by spectrophotometer at 280 nm and confirmed with HPLC. TMG_2 fraction was combined and dried by rotary evaporator and freeze-dryer for structure elucidation and further experiment.

2.16 Mass spectrometry (MS)

Electrospray Ionization-Time of Flight Mass Spectrometry (ESI-TOF MS) profile was performed by a microTOF at the Service Unit of the National Center for Genetic Engineering and Biotechnology. The mixture of methanol: H_2O (1:1 v/v) was used as solvent of sample for analysis.

2.17 Nuclear Magnetic Resonance (NMR)

^1H and ^{13}C NMR spectra were obtained by Miss Wanna Bannarakkul at the Organic Synthesis Research Unit of Department of Chemistry, Faculty of Science Chulalongkorn University. The operation was at 400 MHz for protons and 100 MHz for carbons. The chemical shifts were shown in ppm downfield from the signal of internal standard - tetramethylsilane (TMS).

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2.18 Scavenging activity of the glycoside product to 1,1-diphenyl-2-picrylhydrazyl (DPPH)

Antiradical activity was measured by DPPH assay, according to the method of Biois (1958). One milliliter of 10, 25, 50, 100 and 200 μM of vitamin E analogue (Trolox, TMG and the glycoside product) in ethanol/water (1:1, v/v) were added to 0.25 mL of 0.5 mM DPPH in ethanol. The final volume of 1.25 mL reaction mixture was incubated at room temperature for 20 minutes. The decolorized DPPH was measured at 516 nm for quantification of scavenging activity from this equation.

$$\% \text{ Scavenging activity} = \frac{A_{516} \text{ of control} - A_{516} \text{ of sample}}{A_{516} \text{ of control}} \times 100$$

2.19 Solubility of the glycoside product

Ten milligrams of each analogue of vitamin E were dissolved with 5 μl distilled water in microcentrifuge tube. The mixture was placed on a water bath with shaking devices, which worked at 100 rpm at 25 ^{O}C for an hour. Then, the insoluble part was separated by microcentrifugation at 11,180 x g for 10 min. The remained supernatant was analyzed by HPLC. The solubility of each vitamin E analogue in 1 mL distilled water was calculated from its solubility standard curve.

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CHAPTER III

RESULTS

3.1 Partial purification of CGTase

CGTase from *Paenibacillus* RB01 was obtained after cultivation of this bacterium in Horikoshi's medium at $40^{O}C$ with 250 rpm continuous shaking for 60 hrs. Afterwards, the enzyme in supernatant was separated from cells by centrifugation and collected for partial purification via corn starch adsorption with a slight modification from Kato and Horikoshi (1984), according to section 2.6. After the enzyme was eluted from corn starch, concentrated by viva flow and dialyzed against 50 mM acetate buffer containing 10 mM $CaCl_2$, its activity and amount of protein were determined according to the method described in section 2.8 and 2.9. The partial purified enzyme had a specific activity of 1,825.7 U/mg of protein and the % recovery of CGTase was 83% of the total activity as shown in Table 5. Furthermore, the purity of CGTase was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described in section 2.7. Partial purified enzyme was observed after the gel was stained by Coomassie brilliant blue G-250 (Figure 14, lane 1, 2 and 3).

3.2 TM preparation by chemical synthesis

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), the carboxylic derivative of vitamin E, was strongly reduced to TM (2-hydroxyl-2,5,7,8-tetramethylchroman-6-ol) by $LiAlH_4$ (Figure 10). The suspension was refluxed for 20 hours, followed by treatment with concentrated HCl and extracted by separatory funnel. The reaction mixture was further purified by silica gel column chromatography and the product was checked by TLC and structurally elucidated by ^{1}H-NMR and ^{13}C-NMR. Figure 15 displayed TLC results that showed Trolox and TM spots at R_f values of 0.42

Table 5 Purification of CGTase from *Paenibacillus* sp. RB01

Purification step	Volume (mL)	Total* activity (U)	Total Protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	1,500	22,328	213	105	1	100
Starch adsorption	250	21,612	75.8	285.3	2.7	97
Ultrafiltration	20	18,622	10.2	1825.7	17.4	83

*Dextrinizing activity

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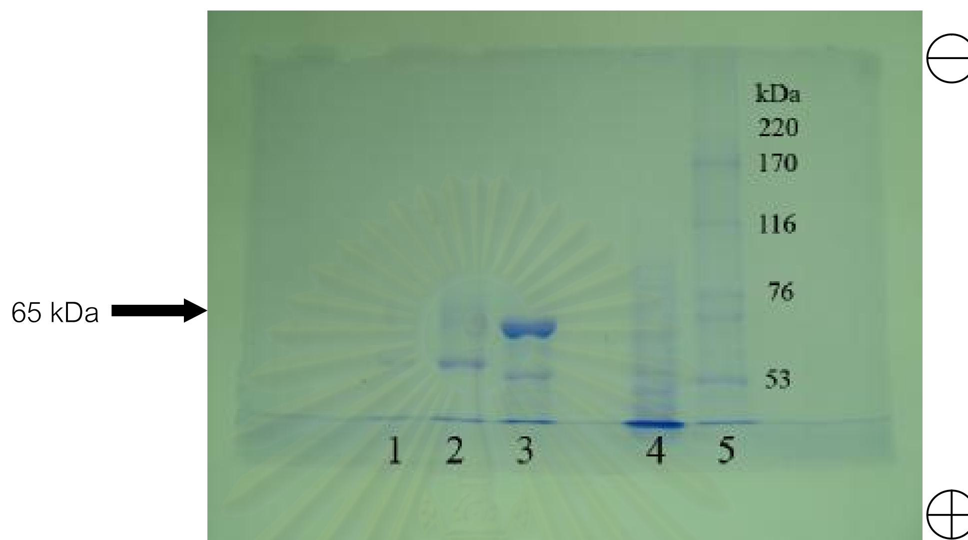

Figure 14. SDS-PAGE of CGTase from *Paenibacillus* sp. RB01.

Lane 1: CGTase (5 μg)

Lane 2: CGTase (10 μg)

Lane 3: CGTase (15 μg)

Lane 4: Crude CGTase (15 μg)

Lane 5: Protein molecular weight markers (myosin 220 kDa, α-2-macroglobulin 170 kDa, β-galactosidase 116 kDa, transferin 76 kDa and glutamate dehydrogenase 53 kDa)

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Figure 15. TLC chromatogram of Trolox and the reduced product, TM, before and after purification (by silica gel column chromatography, ethyl acetate/hexane 7:3 (v/v) as a mobile phase) detected by dipping in $KMnO_4$ solution.

Lane 1: The reaction mixture before purification

Lane 2 – 5: Fraction number 30, 35, 40 and 41, respectively

Lane 6 – 10: Fraction number 43, 45, 42, 60 and 79, respectively

Lane 11: Fraction number 60

Lane 12: Fraction number 67

Lane 13 – 18: Fraction number 69, 70, 71, 73, 75 and 81

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and 0.24, respectively. The ^{1}H NMR dimethyl sulfoxide [(DMSO)-d_6] spectra and the ^{13}C-NMR result of TM were shown in Figure 16 and 17, respectively and the chemical shifts were compared to those from Murase *et al.* report (1997) as shown in Table 6 for ^{1}H-NMR and Table 7 for ^{13}C-NMR. The chemical shifts of TM were in good agreement with the previous report. The ^{1}H NMR spectra showed that the product molecule consisted of hydroxyl group with –OH group on the ethyl functional group (δ = 3.36) and from the ^{13}C NMR spectra, the chemical shifts of the chromanol moiety of TM were essential the same as those of Trolox except that it contained –CH_2-OH group. The yield in mole percent of TM synthesized was 92% (0.8760 g of TM from 1 g of Trolox). The TM produced was then used as a glycosyl acceptor in the following experiment.

3.3 Transglycosylation to TM and TMG

Attempts were initially made to synthesize Tocopherol monoglucoside (TMG) by CGTase from TM and β-cyclodextrin as acceptor and donor, respectively. The three different substrates, maltoheptaose, β-cyclodextrin and soluble starch, were used as glucosyl donors for this experiment. The reaction mixture composed of 0.2 mL of 1% (w/v) TM, 0.624 mL of 1.5% (w/v) donor and 400 U of CGTase was incubated at 40°C for 20 hours. Then, the reaction was stopped, aliquot of which was analyzed by TLC and HPLC. It was expected that TMG should appear as the spot on TLC plate or peak signal in HPLC. Unfortunately, neither spot nor peak was detected by these methods (Figure 18 and 19). Therefore, this main experiment needs to be modified. The Murase *et al.* (1997) method was introduced to synthesize TMG, which was then used as an acceptor to synthesize the TM derivative that contained more than one glucose unit (TMG_n) by transglycosylation reaction of CGTase.

The transglycosylation to TM by α-glucosidase using maltose as a glycosyl donor was performed as described previously in Section 2.10. After the incubation was completed, the aliquot was analyzed by TLC and HPLC as described in Section 2.12. As shown in Figure 20A, one reaction product was detected as a spot on TLC with R_f value of 0.46, which appeared after TM spot (R_f value of 0.94). Figure 20B showed HPLC

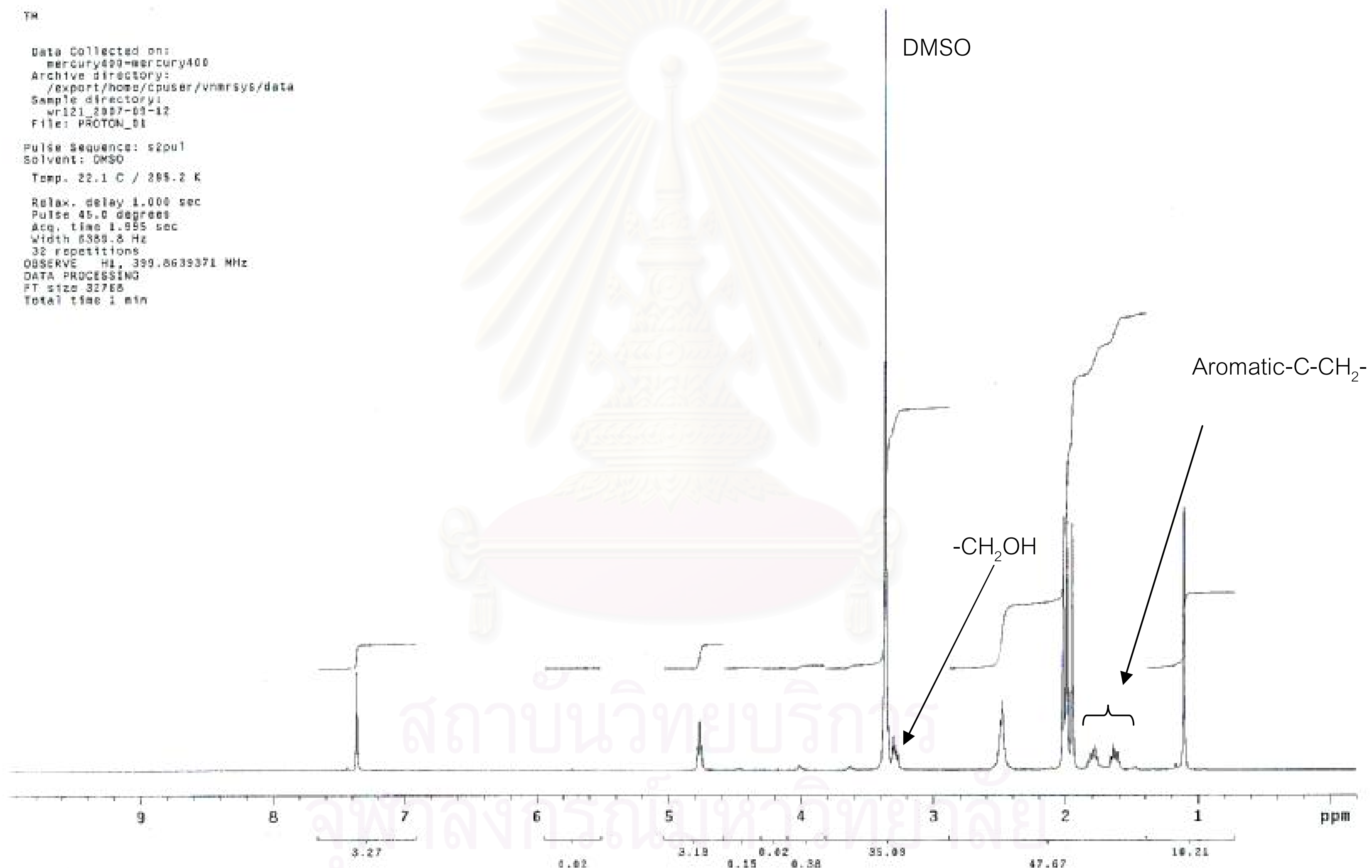

Figure 16. The 400 MHz ^{1}H-NMR spectra of TM.

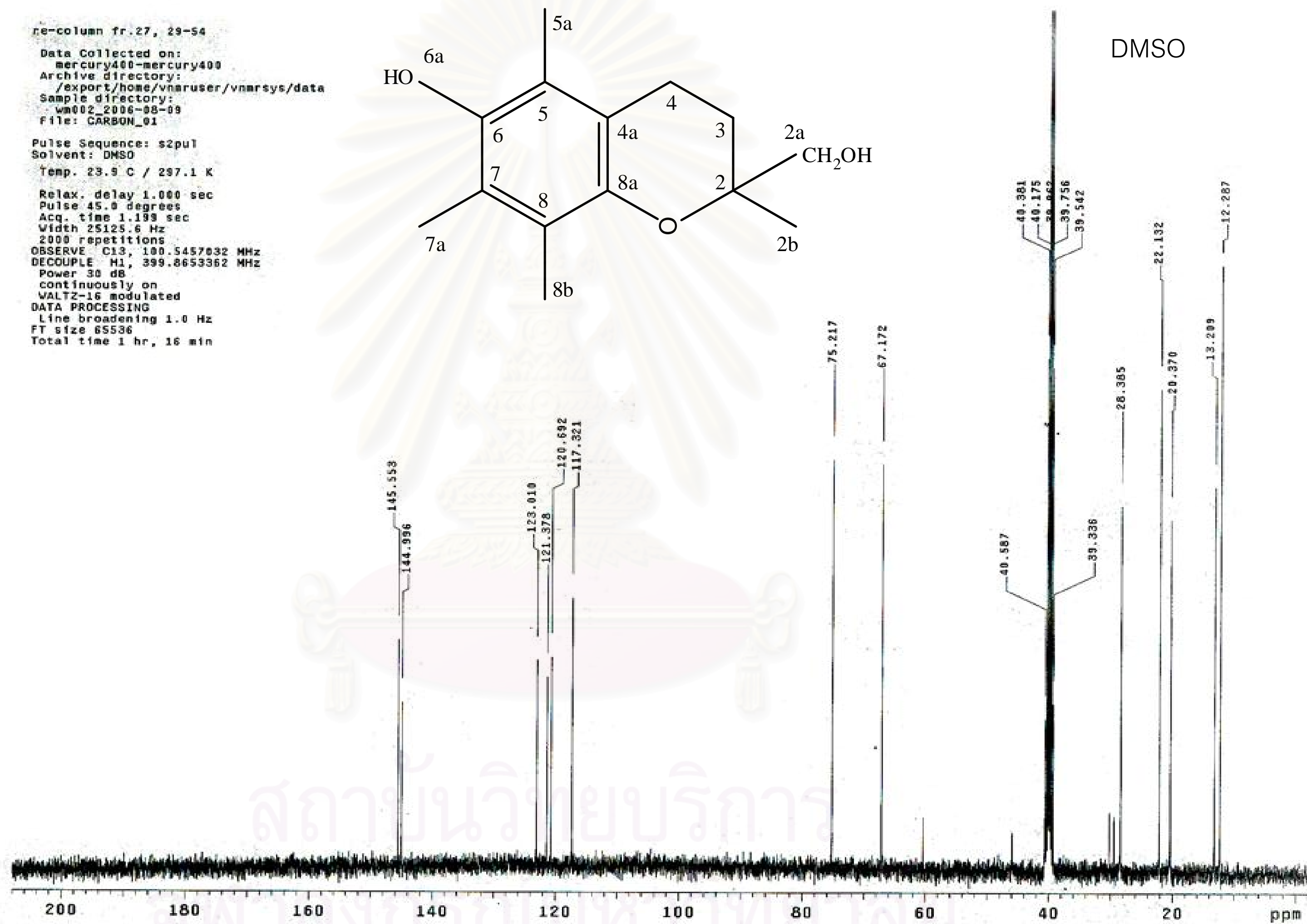

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Figure 17. The 100 MHz ^{13}C-NMR spectra of TM with the molecular structure of TM.

Table 6 ^{1}H Nuclear Magnetic Resonance Chemical shift of (R,S)-form of TM (in parts per million downfield relative to tetramethylsilane)

Chemical Shift of (R,S)-TM in Murase's report (1997)	Chemical Shift of (R,S)-TM in this report
1.14 (s, 3H)	1.12
1.60-1.88 (m, 2H)	1.60-1.68 and 1.76-1.82
1.98 (s, 3H)	1.96
2.02 (s, 3H)	2.00
2.05 (s, 3H)	2.04
2.51 (broad t, 2H)	2.50
3.35 (q, J = 10.7 Hz, 2H)	3.36
4.78 (broad s, 1H)	4.76
7.38 (s, 1H)	7.38

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Table 7 ^{13}C Nuclear Magnetic Resonance Chemical shift of (R,S)-form of TM (in parts per million downfield relative to tetramethylsilane)

Chromanol moiety position	Chemical Shift of (R,S)-TM in Murase's report (1997)	Chemical Shift of (R,S)-TM in this report
2	74.8	75.2
2a	66.8	67.2
2b	21.7	22.1
3	28.0	28.4
4	19.9	20.4
4a	116.9	117.3
5	120.2	120.7
5a	11.8	12.3
6	144.6	145.0
7	121.0	121.4
7a	12.7	13.2
8	122.6	123.0
8a	145.1	145.6
8b	11.8	12.3

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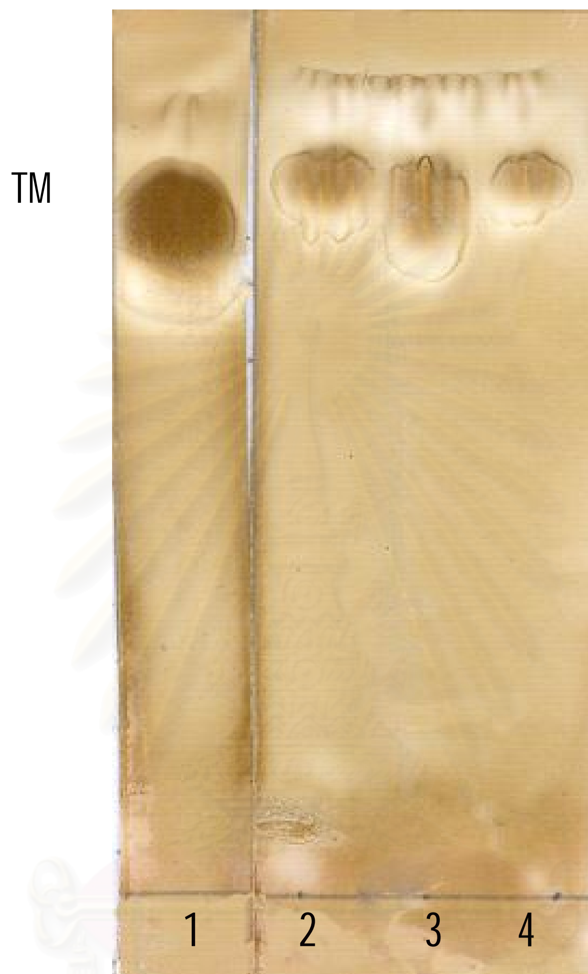

Figure 18. TLC analysis of TM and the reaction mixture containing TM, various donors and CGTase. TLC was performed on silica gel plates with chloroform /methanol/acetic acid/water (40:10:2:1 by vol) as a solvent system. Spots were visualized by dipping in $KMnO_4$ solution. Lane 1: TM as standard, Lane 2: TM with β-cyclodextrin, Lane 3: TM with maltoheptaose, Lane 4: TM with soluble starch.

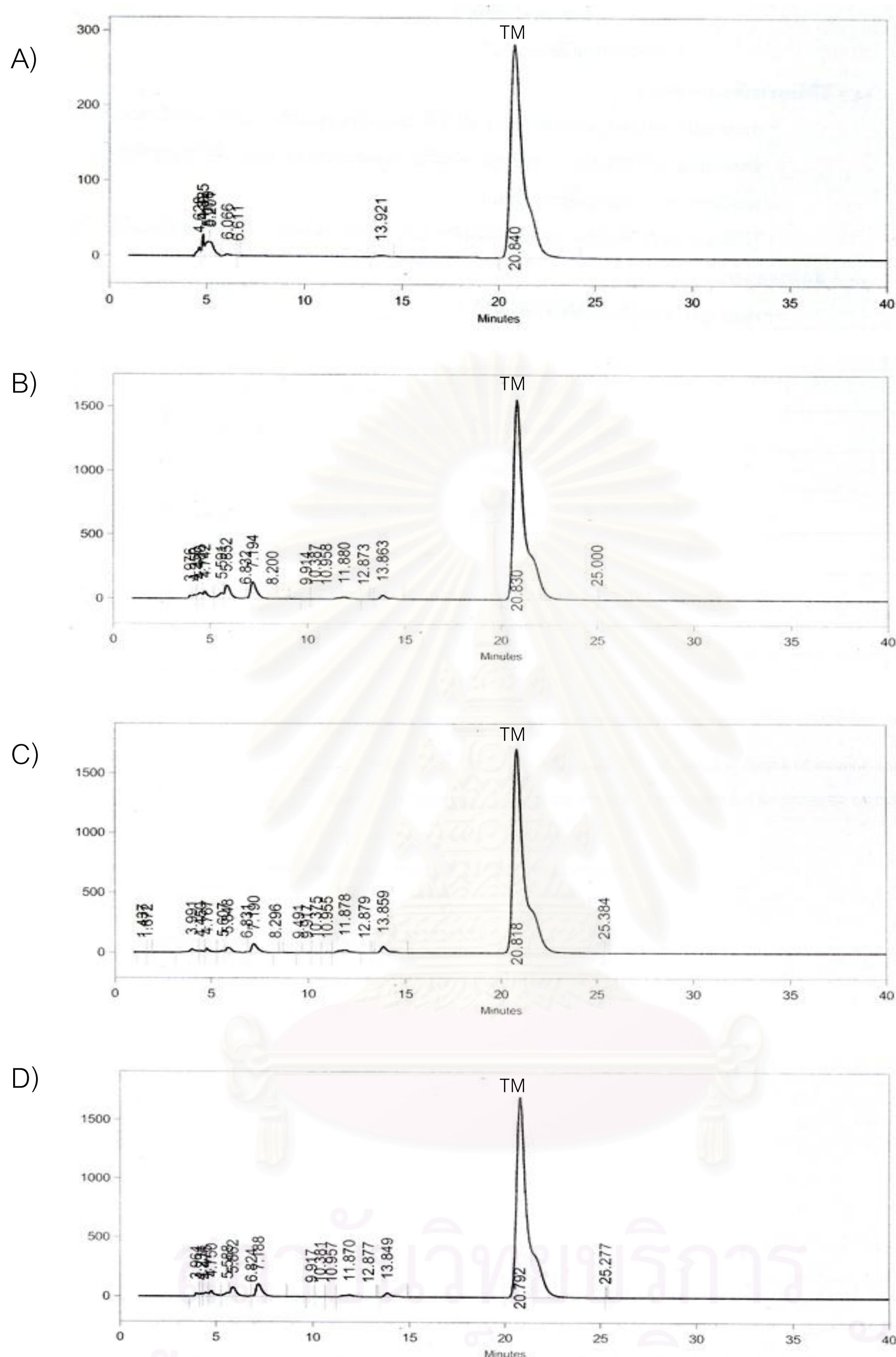

Figure 19. Reversed-phase HPLC chromatogram of TM and the reaction mixture containing TM, various donors and CGTase. HPLC was performed with Inertsil ODS-3 column (4.6 x 250 mm) and eluted with acetonitrile/water (40:60, v/v) at a flow rate of 0.5 mL/min A) TM as standard; B), C) and D) the reaction mixture containing with 1% (w/v) of TM, 400U of CGTase and 1.5 % (w/v) of β-cyclodextrin, maltoheptaose and soluble starch, respectively.

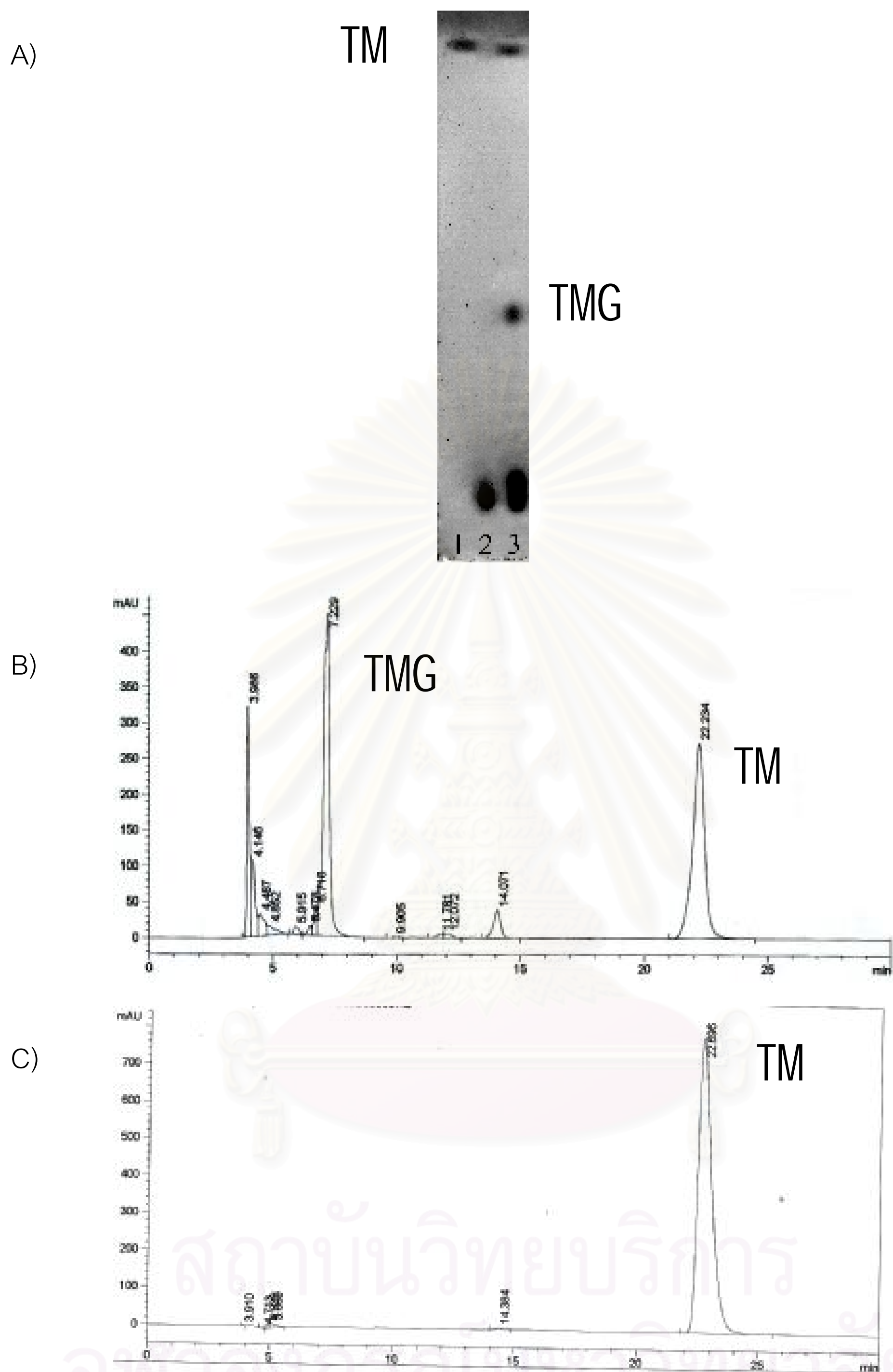

Figure 20. TLC and HPLC analysis of the transglycosylation product from maltose and TM using α-glucosidase. A) TLC was performed by using chloroform /methanol/acetic acid/water (40:10:2:1 by vol) as a solvent system and silica gel as adsorbent. The product was detected by spraying H_2SO_4/methanol (1:4, v/v) on TLC plate before charring. Lane 1: TM as standard, Lane 2: maltose, Lane 3: reaction mixture containing 1% (w/v), 30 U of α-glucosidase and 40% (w/v) maltose. B) Reversed-phase HPLC chromatogram of the reaction mixture containing 1% (w/v) of TM, 30 U of α-glucosidase and 40% (w/v) of maltose. C) Reversed-phase HPLC chromatogram of TM. HPLC was performed with Inertsil ODS-3 column (4.6 x 250 mm) and eluted with acetonitrile/water (40:60, v/v) at a flow rate of 0.5 ml/min.

detected with retention time (R_t) of 7 minutes and appeared before the peak of TM substrate. This was consistent with previous report (Murase *et al.*, 1997). Therefore, it was believed to be TMG.

The production of a novel vitamin E glycoside was then carried out by CGTase using TMG as an acceptor and β-CD as a donor. After the reaction was completed, the reaction mixture was subjected to TLC and HPLC analysis. A novel compound spot was observed at R_f value of 0.22 before TMG spot (R_f value of 0.46) (Figure 21A) and the additional peak of glycoside product was observed at R_t of 5.9 minutes (Figure 21B). In order to assure that the product observed as a spot on TLC and a peak in the HPLC profile was transglycosylation product by the action of CGTase, the reaction mixture without CGTase, reaction mixture without β-CD and reaction mixture with 0 hour incubation with CGTase were used as control experiments. Spots in Figure 22A were visualized by dipping TLC plate in $KMnO_4$ solution for the detection of the unsaturated compound and alcohols (Olivo, 1996), whereas spots in Figure 22B were visualized by spraying with H_2SO_4/methanol (1:6; v/v) followed by heating at 110 ^{O}C for the detection of sugar and glycosyl derivatives. As can be seen in Figure 22 that TMG_n was only synthesized in the reaction mixture containing the acceptor (TMG), donor (β-CD) and CGTase while no products were observed under control conditions. This corresponded with HPLC chromatogram in the Figure 23 where no additional peak was observed under control conditions except for the reaction mixture containing TMG, β-CD and CGTase. From these results, the product was presumed to be TMG_n. In addition, to confirm that the product was a glycoside derivative, the reaction mixture was then treated with glucoamylase (20 U). After addition of glucoamylase to this reaction mixture and overnight incubation at 40 ^{O}C, the peak at R_t of 5.9 minutes disappeared and only the content of TMG was increased (Figure 24). Thus, the peak at R_t of 5.9 minutes was confirmed to be the conjugate of TMG and oligosaccharides corresponding to TMG_n.

A)

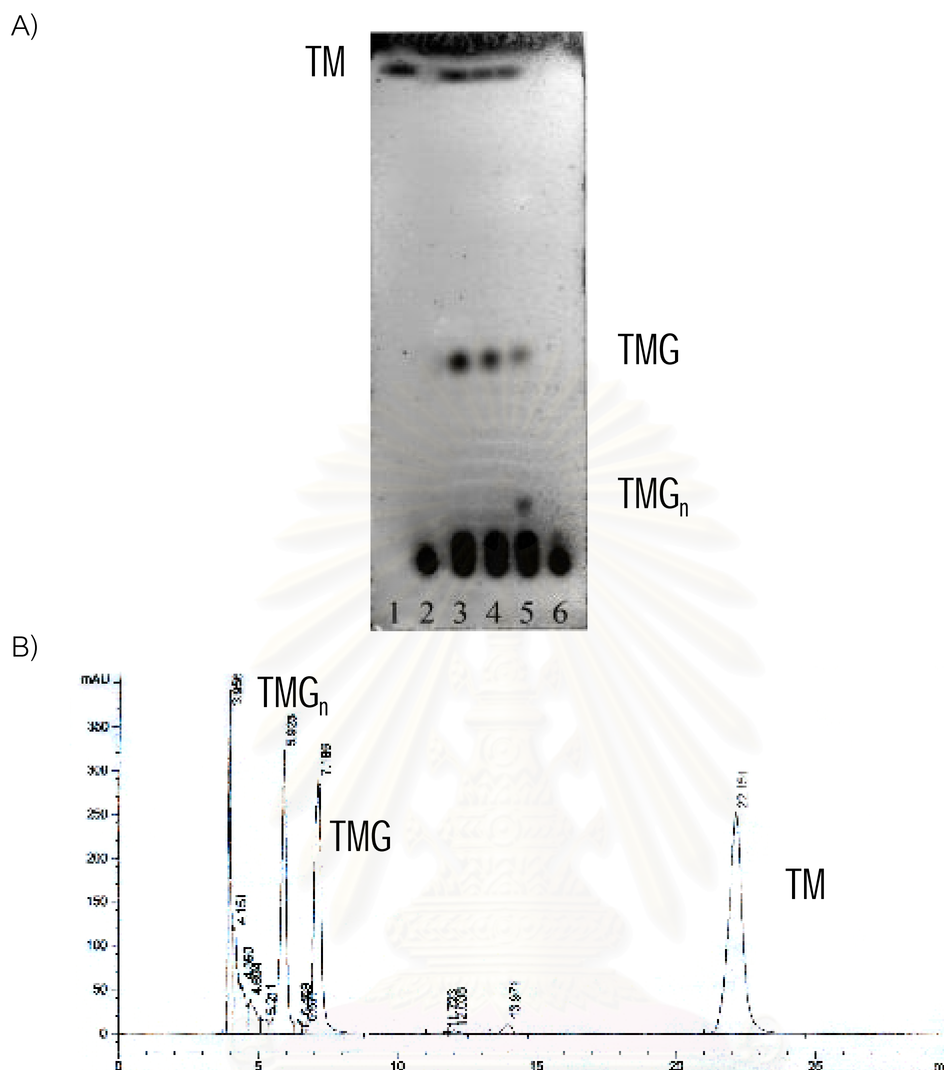

Figure 21. TLC (A) and HPLC (B) analysis of the transglycosylation product from maltose to TM by α-glucosidase and β-cyclodextrin to TMG by CGTase. TLC was performed by using chloroform/methanol/acetic acid/water (40:10:2:1 by vol) as a solvent system and silica gel as adsorbent. The product was detected by spraying H_2SO_4/methanol (1:4, v/v) on TLC plate before charring. Lane 1: TM as standard, Lane 2: maltose, Lane 3: reaction mixture containing 1% (w/v) of TM, 30 U of α-glucosidase and 40% (w/v) of maltose, Lane 4: reaction mixture containing 30% (v/v) mixture of TMG (from 3), 100 U of CGTase and 1.5% (w/v) β-cyclodextrin at 0 hour incubation with CGTase, Lane 5: reaction mixture containing 30% (v/v) mixture of TMG (from 3), 100 U of CGTase and 1.5% (w/v) β-cyclodextrin after 16 hour incubation with CGTase, Lane 6: β-cyclodextrin. HPLC was performed with Inertsil ODS-3 (40:60, v/v) as a mobile phase at a flow rate of 0.5 mL/min. B) Reversed-phase HPLC chromatogram of the reaction mixture containing 30 % (v/v) mixture of TMG (from 3), 100 U of CGTase and 1.5% (w/v) β-cyclodextrin . after 16 hours incubation with CGTase.

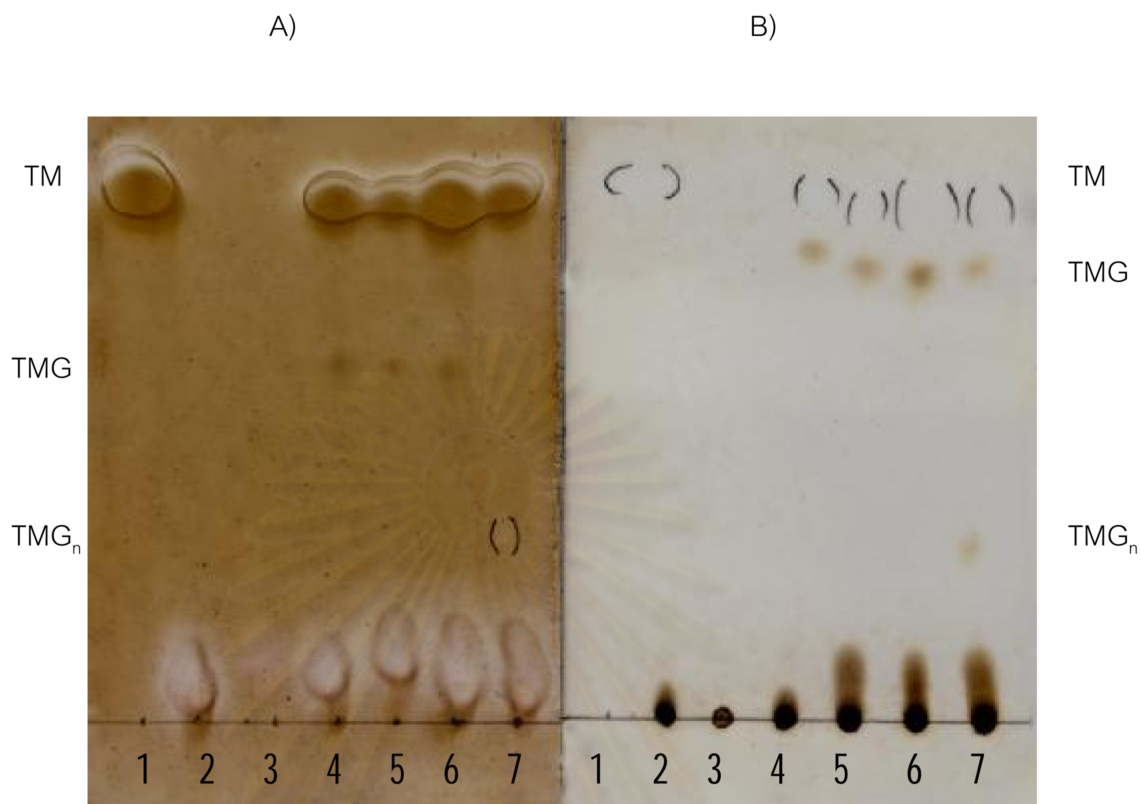

Figure 22. TLC analysis of the transglycosylation products by CGTase. TLC was carried out using chloroform/methanol/acetic acid/water (40:10:2:1 by vol) as a solvent system and silica gel as adsorbent. The product was detected by dipping TLC plate in $KMnO_4$ solution (A) and by spraying H_2SO_4/methanol (1:6, v/v) on TLC plate before charring (B). Lane 1: TM as standard, Lane 2: maltose, Lane 3: β-cyclodextrin, Lane 4: reaction mixture without CGTase, Lane 5: reaction mixture without β-CD, Lane 6: reaction mixture at 0 hour incubation with CGTase, Lane 7: reaction mixture after 16 hours incubation with CGTase.

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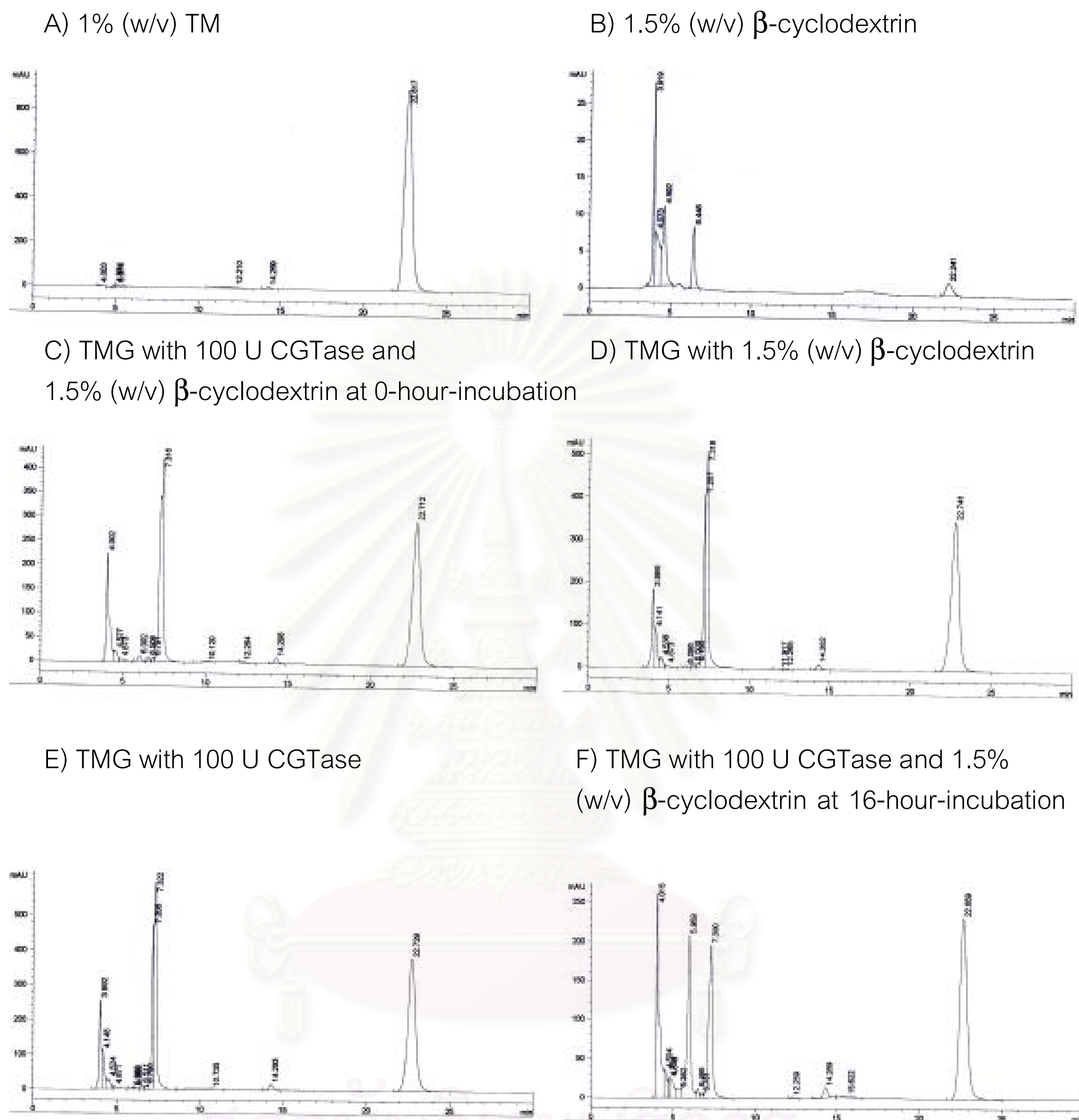
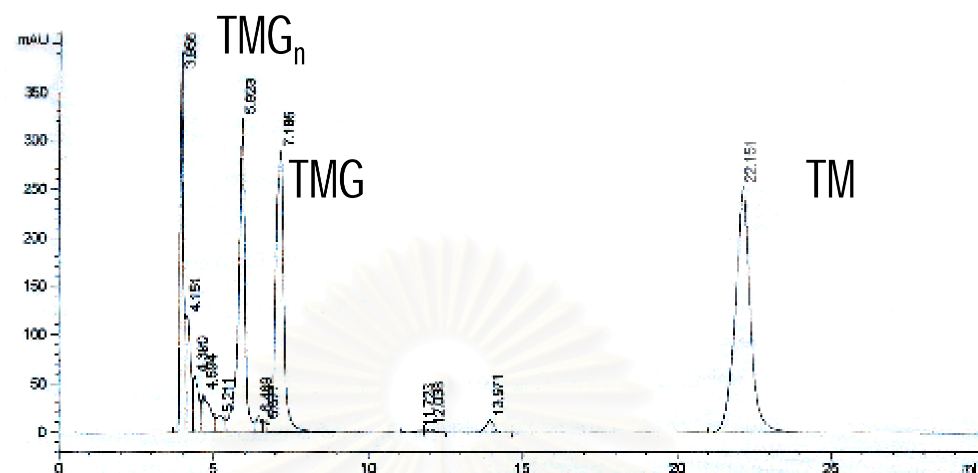


Figure 23. Reversed-phase HPLC chromatogram of the transglycosylation products by CGTase. HPLC was performed with Inertsil ODS-3 column (4.6 x 250 mm) and acetonitrile/water (40:60, v/v) was used as a mobile phase at a flow rate of 0.5 mL/min. A) TM as standard, B) β-cyclodextrin, C) reaction mixture at 0 hour incubation with CGTase, D) reaction mixture without CGTase, E) reaction mixture without β-CD, F) reaction mixture after 16 hours incubation with CGTase.

A) Before treatment with glucoamylase



B) After treatment with glucoamylase

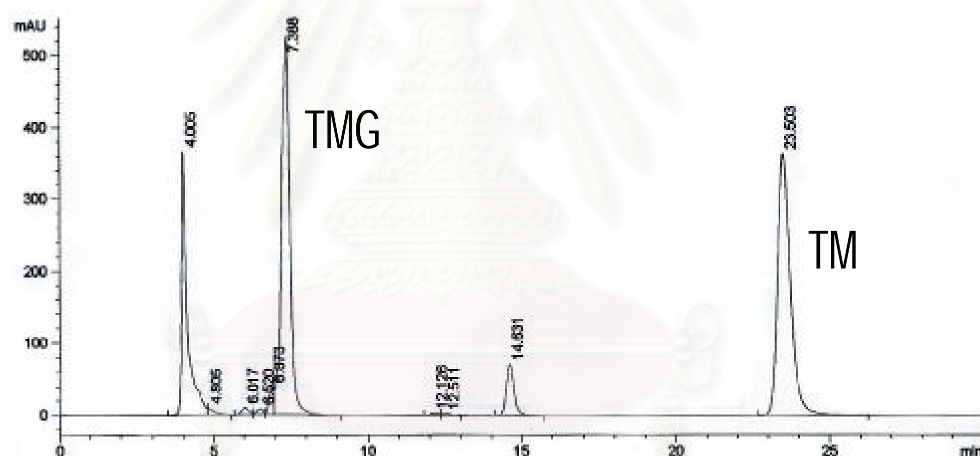


Figure 24. A) Reversed-phase HPLC chromatogram of the transglycosylation product from β-cyclodextrin to TMG by CGTase. B) Effect of glucoamylase on the digestion of TMG_n. HPLC was performed with Inertsil ODS-3 column (4.6 x 250 mm) and eluted with acetonitrile/water (40:60, v/v) at a flow rate of 0.5 mL/min.

3.4 Optimization of the production of vitamin E glycoside

In this part, the experiment was carried out to find the appropriate conditions for the synthesis of TMG_n. To obtain large-scale production of TMG_n, several parameters involved in the reaction including temperature, pH, incubation time, the substrate and enzyme concentration were optimized.

3.4.1 Effect of temperature

In order to investigate the effect of temperature on TMG_n synthesis, five different conditions of which were determined as expressed in Section 2.13.1. The temperatures in the range of 30-70°C were used. The concentrations of both TM and β-CD were 1 % (w/v) and these precursors were allowed to mix with 100 U of CGTase. After the incubation reached 20 hours, the reaction mixture was analyzed by HPLC. The amount of TMG_n formed was analyzed by comparing the retention time to that of the TMG and was calculated on the basis of the ratio of peak area. Since the TMG produced from the first transglycosylation reaction (using α-glucosidase to catalyze the transglycosylation to TM from maltose) had not been purified, the ratio of peak area of TMG_n/TMG was then used for the analysis of TMG_n production. It was found that the CGTase could catalyze the transglycosylation to TMG from β-CD at the temperature ranging from 30-50°C with the highest amount of TMG_n formed at 50°C. At higher temperature the amount of TMG_n decreased rapidly (Figure 25). Thus, the optimal temperature of 50°C was chosen for TMG_n production.

3.4.2 Effect of pH

The effect of pH on CGTase-catalyzed coupling reaction from β-CD to TMG for TMG_n production was investigated. The nine conditions of different pH values in three kinds of buffer solution were employed in this experiment. The three diverse buffers consisting of acetate buffer (pH 4.0, 5.0 and 6.0), phosphate buffer (pH 6.0, 7.0 and 8.0) and Tris-glycine buffer (pH 8.0, 9.0 and 10.0) with the same concentration were used as

the main environment of the reaction mixture. The protocol was done as shown in Section 2.13.2 with optimum temperature of 50°C. The amount of TMG_n was analyzed by the same method as described in the previous section. As shown in Figure 26, the amount of TMG_n was maximum at the reaction pH of 6.0.

3.4.3 Effect of incubation time

The incubation time was varied from 0 - 48 hours with optimum temperature of 50°C and the reaction pH of 6.0. After the experiment was carried out as described in section 2.13.3, the reaction mixture was analyzed by HPLC. The production of TMG_n was also analyzed by the ratio of peak area of TMG_n/TMG. Figure 27 shows that the highest amount of TMG_n formed was obtained at 4 hour-incubation time. The prolongation of the incubation time did not increase the TMG_n production.

Thus, the optimal conditions for TMG_n production by CGTase were to carry out the reaction at 50°C, pH 6.0 for 4 hours. Next, the TMG needed to be purified prior to the investigation for the optimal concentration of donor, acceptor and enzyme on the production of TMG_n.

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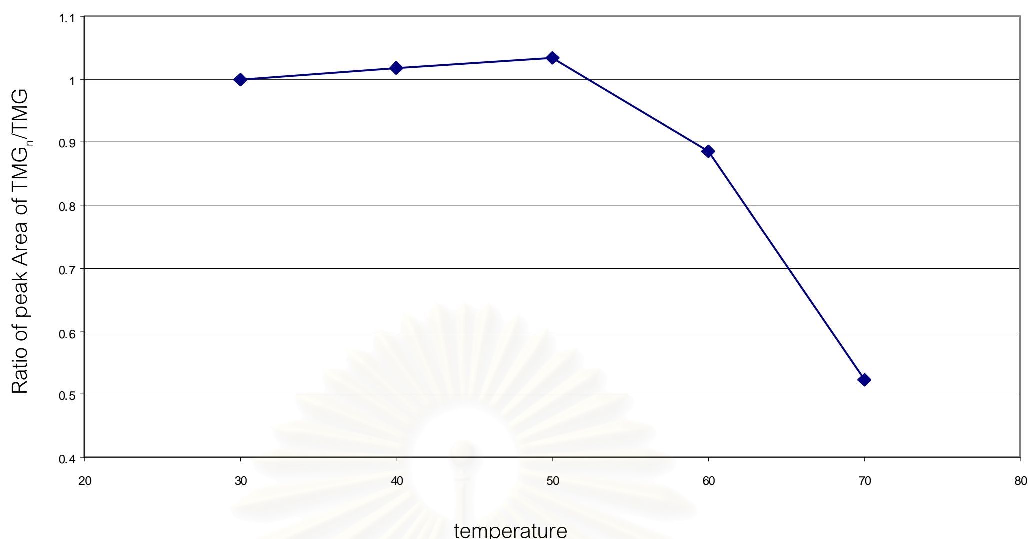

Figure 25. Effect of temperature on TMG_n production by CGTase. The CGTase (100 U) was incubated with a mixture containing TMG (from the transglucosylation reaction by α-glucosidase) and 1.5% (w/v) β-CD at indicated temperatures for 16 hours.

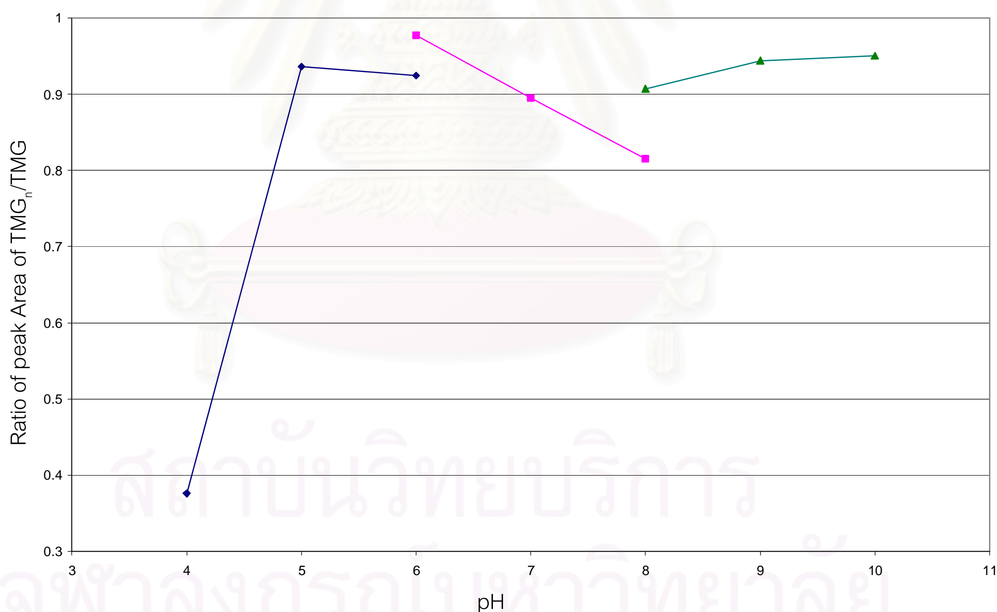

Figure 26. Effect of pH on TMG_n production by CGTase. The CGTase (100 U) was incubated with a mixture containing TMG (from the transglucosylation reaction by α-glucosidase) and 1.5% (w/v) β-CD at different pH values at 50°C for 16 hours. Acetate buffer (◆), phosphate buffer (■) and Tris-glycine buffer (▲) were used.

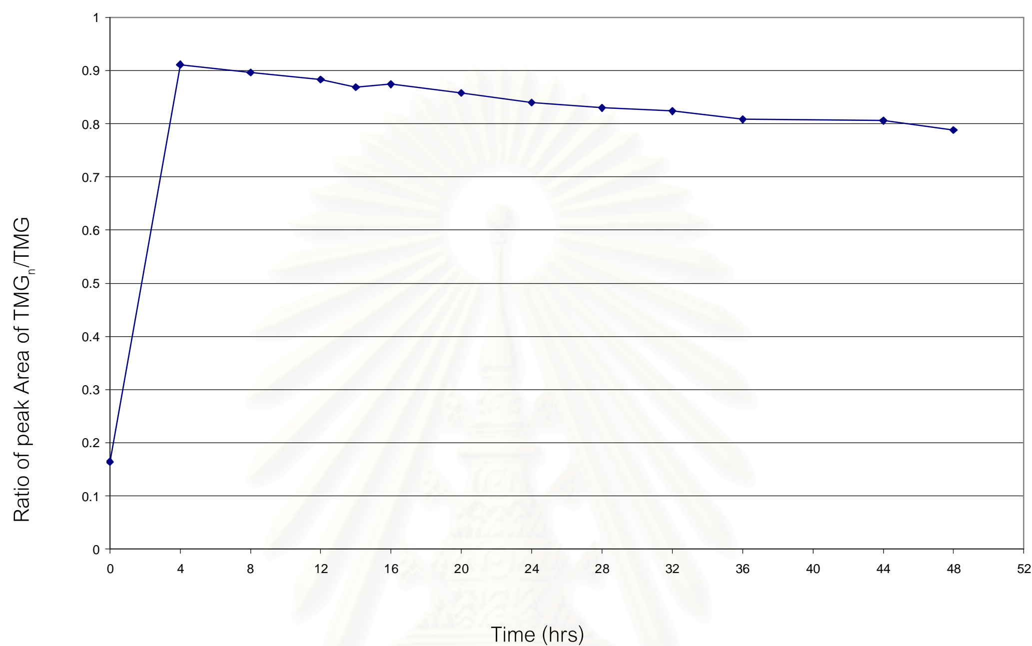

Figure 27. Time course of TMG_n production with CGTase. The CGTase (100 U) was incubated with a mixture containing TMG (from the transglucosylation reaction by α-glucosidase) and 1.5% (w/v) β-CD at 50°C, pH 6.0. The reaction was terminated by boiling at indicated time.

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3.5 Purification of TMG

TMG production was performed according to the method of Murase *et al* (1997). After the incubation was completed, the reaction mixture was combined and concentrated to purify. The solution was passed through Amberlite XAD-4. First, 30% (v/v) methanol was applied to the column to eliminate DMSO and sugars, which were checked by TLC following the condition described in Section 2.12.1. DMSO spot was white (did not burn after charring) and sugars showed black spots on TLC plate (Figure 28). TMG and TM were then eluted after 80% (v/v) methanol was used. The profile of TMG purification was created by 280 nm spectrophotometer observation (Figure 29). The fraction number 153, 175, 181, 183, 201 and 245 were selected for the identification by HPLC according to Section 2.12.2 and the boundary between TMG and TM was marked (Figure 29A). TMG fraction was combined and the solvent was evaporated. After the rest of water was removed by freeze-dryer, TMG was kept and used for the production of TMG_n and structure elucidation. The yield (in mole percent) of TMG synthesis was calculated from the ratio of the moles of TMG after purification to the moles of TM before synthesis. Purified TMG in grams (0.3191 g) was divided by its molecular mass (about 398 from the Section 3.8.1) gave the result of TMG in mole (0.801 mmol). Initial moles of TM (2.116 mmol) were calculated from initial TM in grams (0.500 g) divided by its molecular mass (about 236 from calculation). Therefore, yield of TMG production was 37.85%.

3.6 Large scale production of TMG_n

After TMG was purified, it was used as an acceptor in a large-scale production of TMG_n. However, the appropriate concentrations of CGTase and donor and acceptor needed to be investigated first.

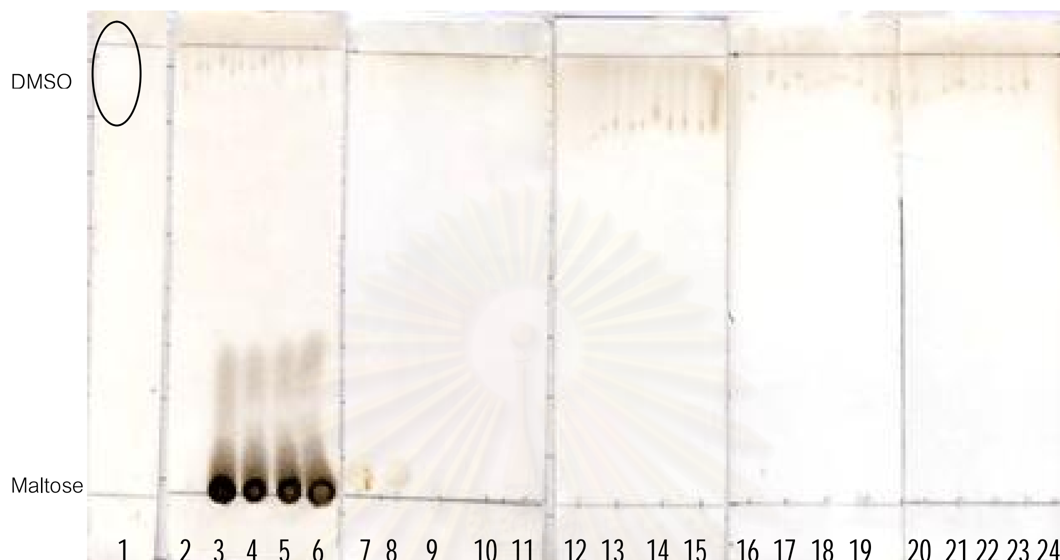

Figure 28. TLC analysis of washed DMSO and maltose by Amberlite XAD-4 purification using 30% (v/v) methanol as a mobile phase. TLC was carried out using chloroform/methanol/acetic acid/water (40:10:2:1 by vol) as a solvent system and silica gel as adsorbent. The product was detected by spraying H_2SO_4/methanol (1:4, v/v) on TLC plate before charring. Lane 1: DMSO as standard, Lane 2-6: fraction number 5, 10, 15, 20 and 25 respectively, Lane 7-11: fraction number 30, 35, 40, 45 and 50, respectively, Lane 12-24: fraction number 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110 and 119, respectively.

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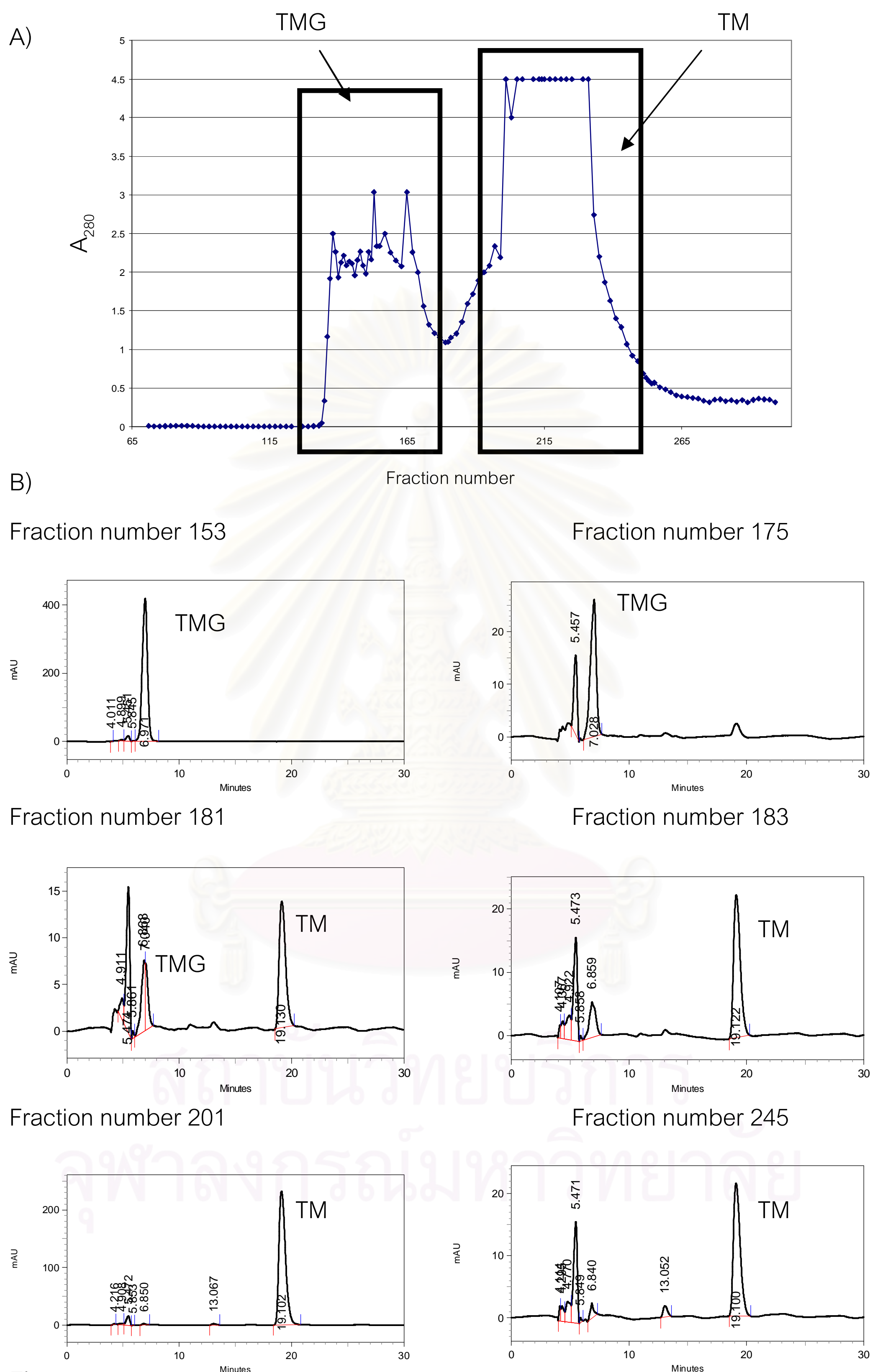

Figure 29. A) The profile of TMG purification by Amberlite XAD-4 (2 cm X 40 cm), which was equilibrated with 30%(v/v) methanol and eluted with 80% (v/v) methanol at a flow rate of 0.5 mL/min. B) HPLC chromatogram of the chosen fraction after purification to identify the profile of purification. HPLC was performed with Inertsil ODS-3 column (4.6 x 250 mm) and eluted with acetonitrile/water (40:60, v/v) at a flow rate of 0.5 ml/min.

3.6.1 Effect of enzyme concentration

The optimization of the TMG_n production for a higher yield was performed by varying amount of CGTase. Various amounts of CGTase concentration (0-150 U, determined with dextrinizing activity) were tested. This experiment used purified TMG as an acceptor. The reaction mixture containing 1% (w/v) TMG, 1.5% (w/v) β-CD was incubated with CGTase at 50°C, pH 6.0 for 4 hours. The production of TMG_n was analyzed by peak area of TMG_n. The CGTase of 100 units gave the highest amount of TMG_n and therefore, was used for further experiment (Figure 30). At higher concentration of CGTase did not produce more TMG_n.

3.6.2 Effect of substrate concentration

To determine the effect of donor and acceptor on TMG_n production, the β-CD concentrations were varied and the TMG concentration was fixed at final concentration of 1% (w/v). The effect of β-CD concentrations on the synthesis of TMG_n is shown Figure 31. It can be seen that the amount of TMG_n formed depended on the donor concentration and β-cyclodextrin at a final concentration of 1.5% (w/v) was appropriate to be used in the next experiment. The effect of TMG concentration on the synthesis of TMG_n is shown in Figure 32. Although the amount of TMG_n increased as the concentration of TMG increased and reached maximum at 1.5% (w/v) of TMG, the production yield was found to be highest when TMG at a final concentration of 0.5% (w/v) was used.

After the condition for transglycosylation of β-CD to TMG by CGTase was completely found, the reaction was performed under these optimal conditions (0.5% (w/v) of TMG, 1.5% (w/v) of β-CD, 100 U of CGTase, pH 6.0, 50 ^{O}C, 4 hours). The reaction mixture was analyzed by HPLC and transglycosylation yield (calculated as described in Section 2.14) was found to be 47.17%.

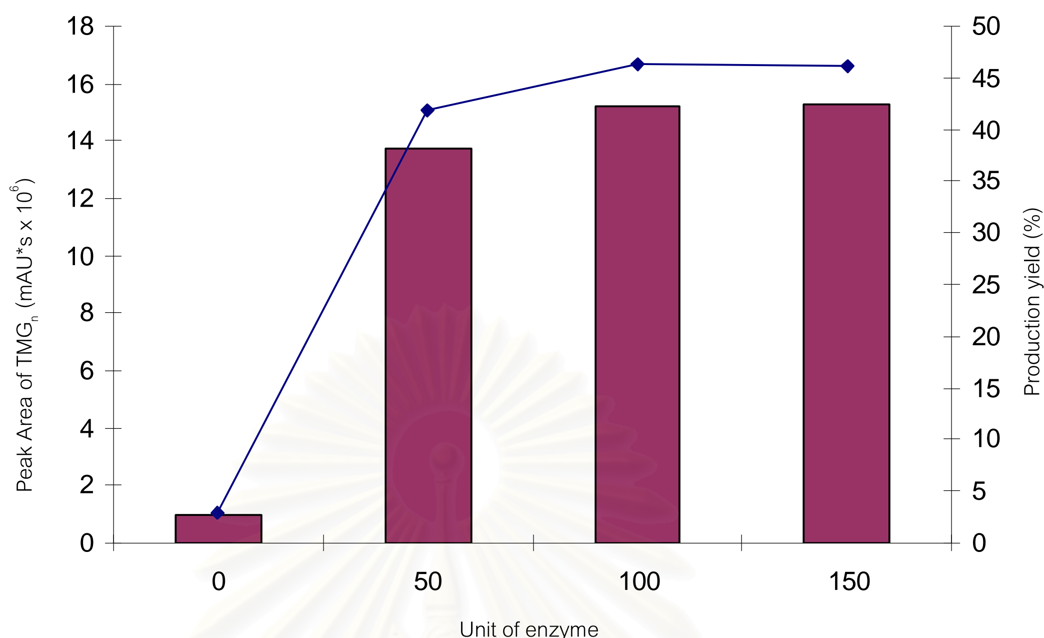

Figure 30. Effect of enzyme concentration on the production of TMG$_n$. The reaction mixture containing 1% (w/v) of TMG and 1.5% (w/v) β-cyclodextrin was incubated with CGTase solution (0-150 U), at 50°C, pH 6.0 for 4 hours.

Production yield (%) Peak area of TMG$_n$

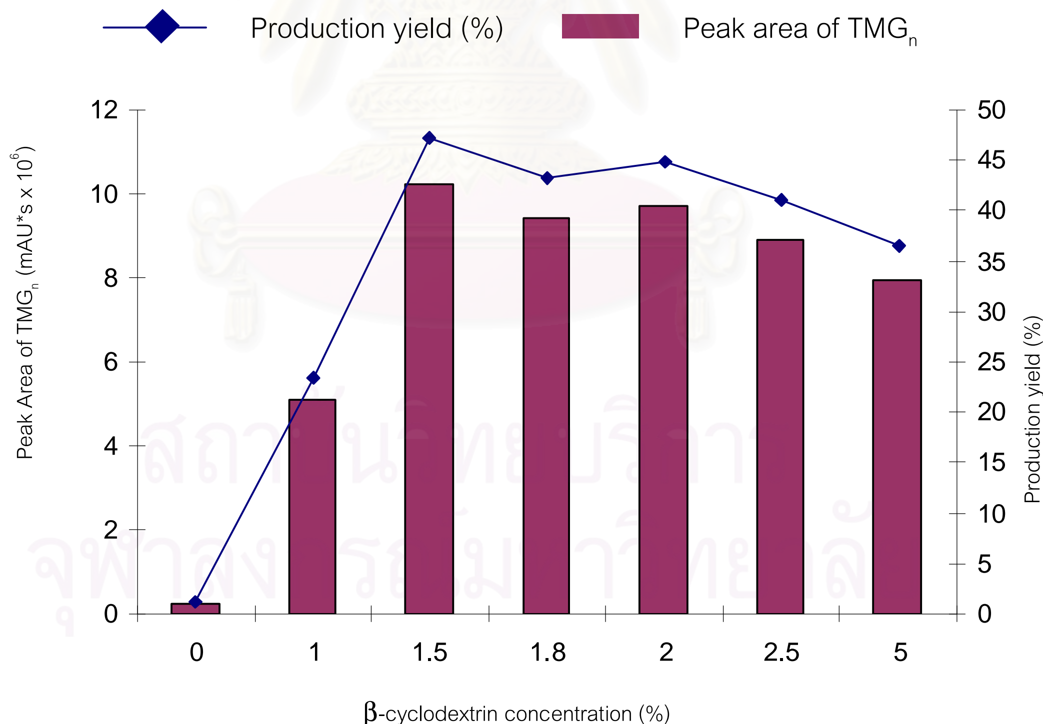

Figure 31. Effect of β-cyclodextrin concentration on the synthesis of TMG$_n$. The reaction mixture containing 1% (w/v) TMG, various amount of β-cyclodextrin (0-5%, w/v) was incubated with 100 U of CGTase at 50°C, pH 6.0 for 4 hours .

Production yield (%) Peak area of TMG$_n$

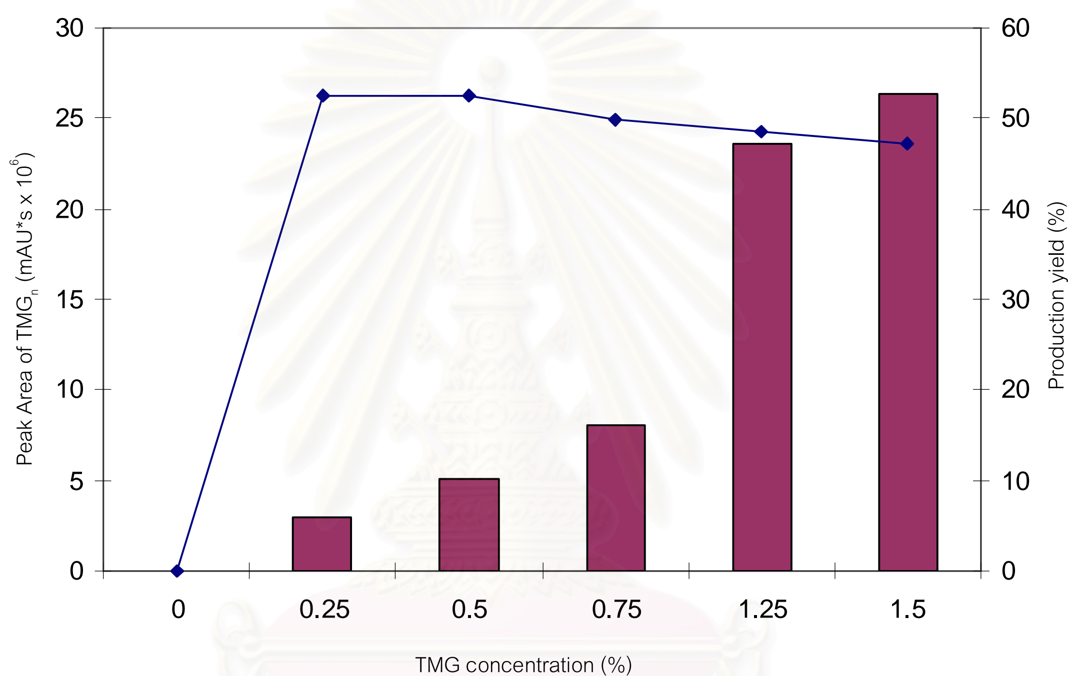

Figure 32. Effect of TMG concentration on the synthesis of TMG$_n$. The reaction mixture containing 1.5% (w/v) β-cyclodextrin and various amount of TMG (0-1.5%, w/v) was incubated with 100 U of CGTase at 50°C, pH 6.0 for 4 hours.

Production yield (%) Peak area of TMG$_n$

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3.7 Purification of TMG_n

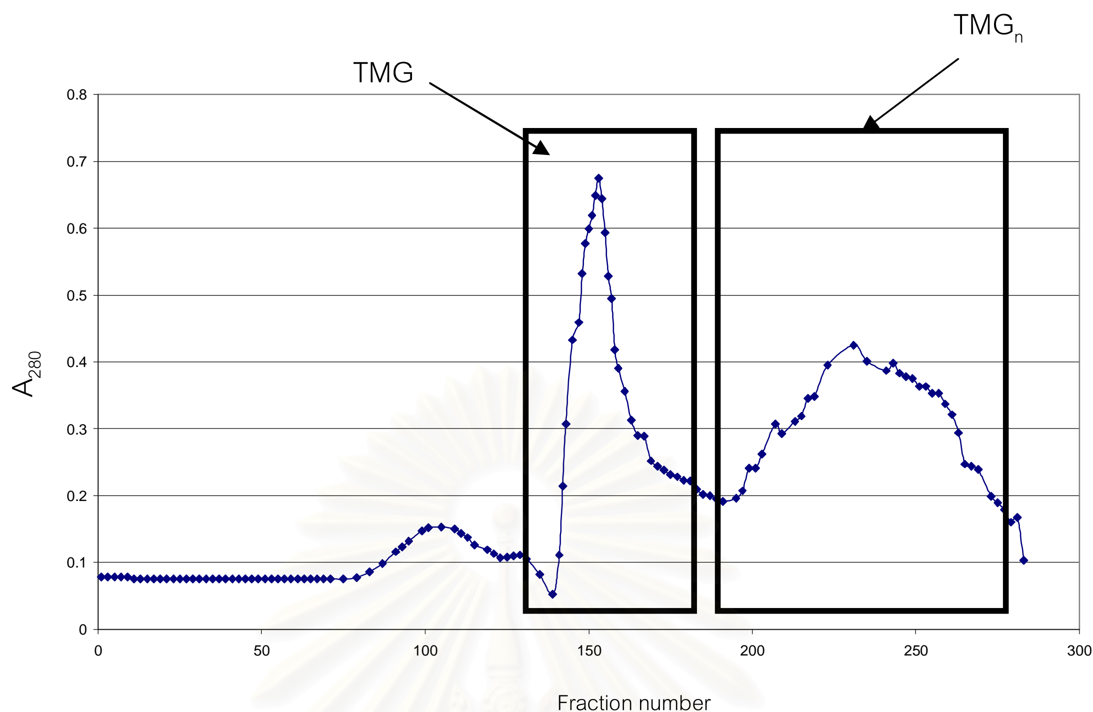
After the reaction conditions were optimized, the TMG_n was then produced in large scale and was purified by silica gel column chromatography as described in Section 2.15.4. The fractions were collected and measured for A_{280} to construct the purification profile (Figure 33). Fraction number 105, 140, 231, 283 were identified by HPLC in order to mark the boundaries of product combination. Then, the solvent was evaporated to obtain the dried glycoside product which was used for structure elucidation. The properties of product such as the solubility in water and scavenging activity will also be investigated. The yield of TMG_n production (in mole percent) was calculated by the same way as described previously. Purified TMG_n in grams (0.0593 g) was divided by its molecular mass (about 560 from the Section 3.6.1) gave the result of TMG in mole (0.106 mmol). Initial moles of TMG (0.603 mmol) were calculated from initial TM in grams (0.240 g) divided by its molecular mass (about 398 from the Section 3.6.1). In conclusion, large-scale TMG_n synthesis had the total yield in mol percent of 17.57% after silica gel purification.

3.8 Characterization of TMG and glycoside product

After the large-scale production and purification of TMG_n had been done and analyzed by HPLC. The purified TMG and TMG_n were subjected to ESI-TOF-MS and ^{13}C-NMR to determine the molecular mass and molecular structure, respectively.

3.8.1 Molecular weight of TMG and TMG_n by MS

TMG and glycoside product were analyzed for their mass as described in Section 2.16 with the positive mode. The molecular ion $[M+Na]^+$ of TMG displayed at m/z 421 (398 plus 23 of sodium molecule), which was in good agreement with previous report (m/z 398 from Murase *et al*, 1997) (Figure 34). The molecular ion $[M+Na]^+$ of TMG_n exhibited at m/z 583 (m/z 560 plus 23 of sodium molecule), which was consistent with the calculated value of 560 from its chemical structure ($C_{20}H_{30}O_8$ plus $C_6H_{10}O_5$) and was in good agreement with TMG plus one glucoside moiety (Figure 35). Therefore, this novel vitamin E glycoside was possibly TMG attached with one glucosyl moiety (TMG_2).



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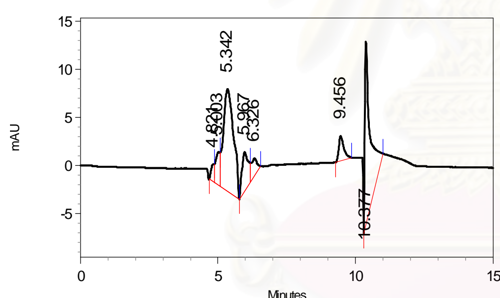

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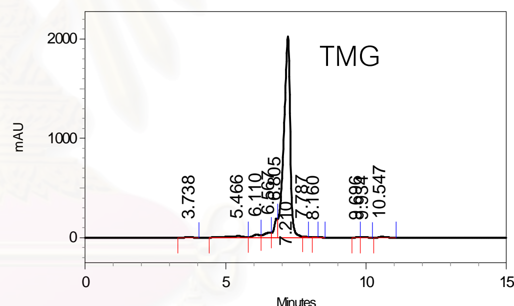

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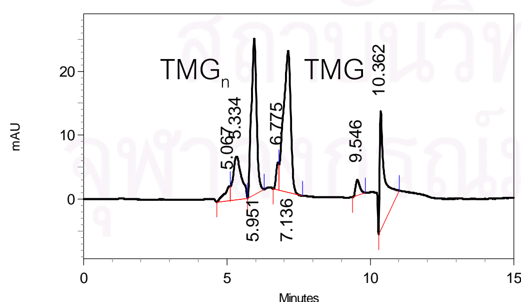

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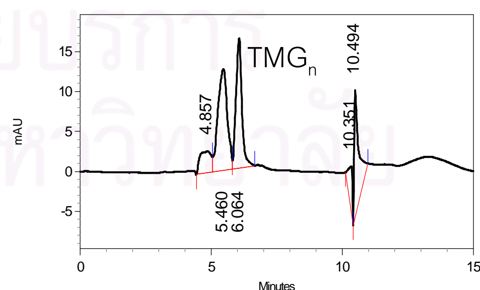

Figure 33. A) The profile of TMG_n purification by silica gel column chromatography using ethyl acetate/methanol (6:1, v/v) as a mobile phase. B) HPLC chromatogram of the chosen fraction after purification to identify the profile of purification. HPLC was performed with Inertsil ODS-3 column (4.6 x 250 mm) and eluted with acetonitrile/water (40:60, v/v) at a flow rate of 0.5 mL/min.

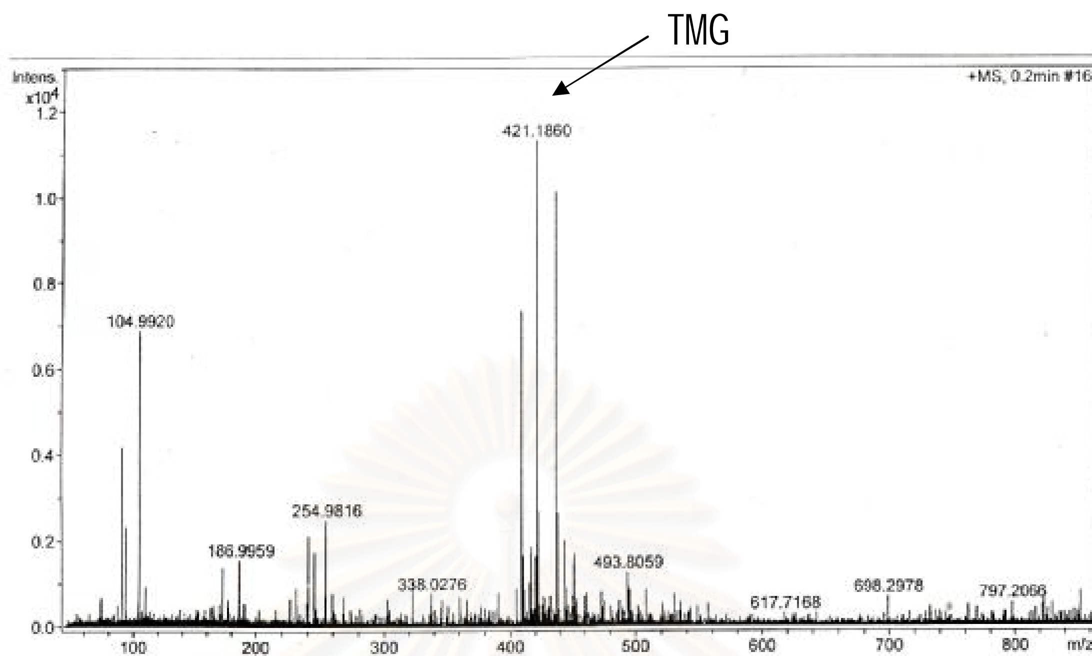

Figure 34. ESI-TOF mass spectra of TMG.

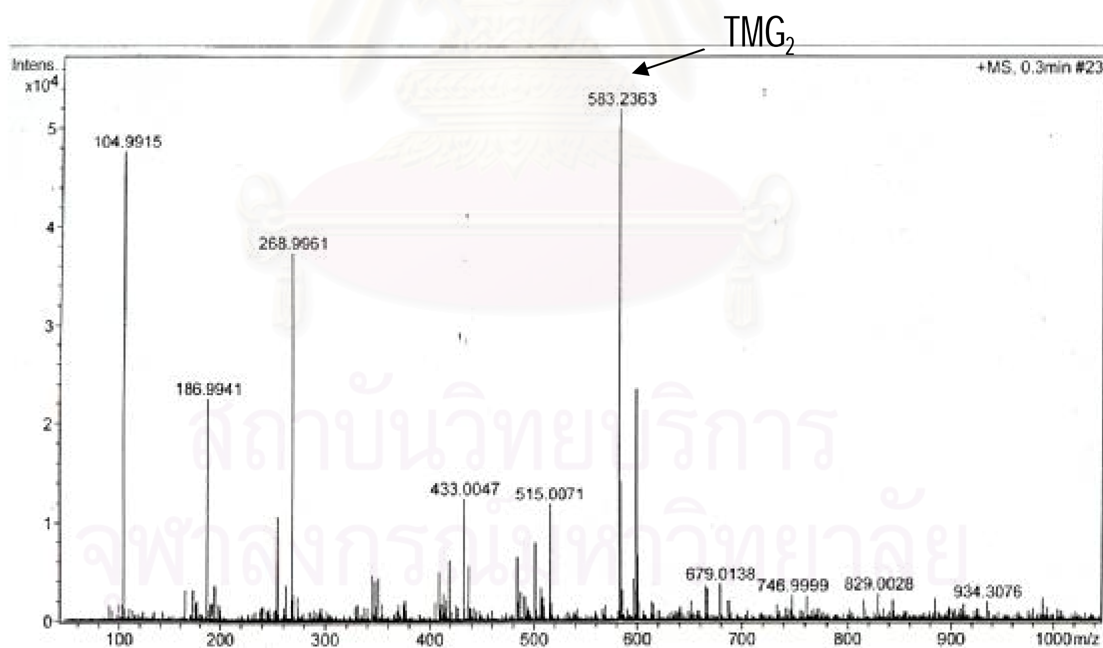

Figure 35. ESI-TOF mass spectra of TMG_2.

3.8.2 Structure elucidation of TMG and glycoside product by NMR

^{1}H and ^{13}C NMR spectra of TMG and glycoside product were obtained after the experiment was performed as described in Section 2.17. The DMSO-d_6 was used to dissolve both compounds. The one-dimension ^{1}H NMR spectra of TMG and TMG_n are displayed in Appendix 4 and 5, respectively. However, the structure of TMG_n could not be resolved only by ^{1}H NMR spectrometer due to the similar environments of many of the protons, ^{13}C NMR analyses were also performed. ^{13}C NMR spectra of TMG and TMG_n are shown in Figure 36 and Figure 37, respectively. Chemical shifts of TMG_n, TMG and TM were compared with those of TMG reported by Murase *et al.* (1997), as shown in Table 8. The α-configuration of the D-glucosyl residue in the TMG was confirmed by the C-1 of sugar signal at δ = 99.3 which was found to be similar to previous report (δ = 98.8). Other chemical shifts of TMG in our experiment were the same as those of TMG from Murase *et al.* report (1997) except for the C-4 shift which was slightly shifted upfield (2.4 ppm) relative to that of TM. Therefore, the structure of TMG was identified as 2-(α-D-glucopyranosyl)methyl-2,5,7,8-tetramethylchroman-6-ol which corresponded to TMG from Murase *et al.* report (1997). The TMG_n spectra were similar to those of TM and TMG except the C-2a shift, which was shifted upfield (2.4 ppm) relative to that of TMG. C-1 of sugar moiety showed the 2 chemical shifts (δ = 101.2 and 98.9) which could refer to as α-maltosyl residue (Webb, 1982). From these data, TMG_n was identified as α-D-glucopyranosyl-(1$\rightarrow$4)-2-(α-D-glucopyranosyl)methyl-2,5,7,8-tetramethylchroman-6-ol (TMG_2). The structures of TMG and TMG_2 are shown in Figure 38.

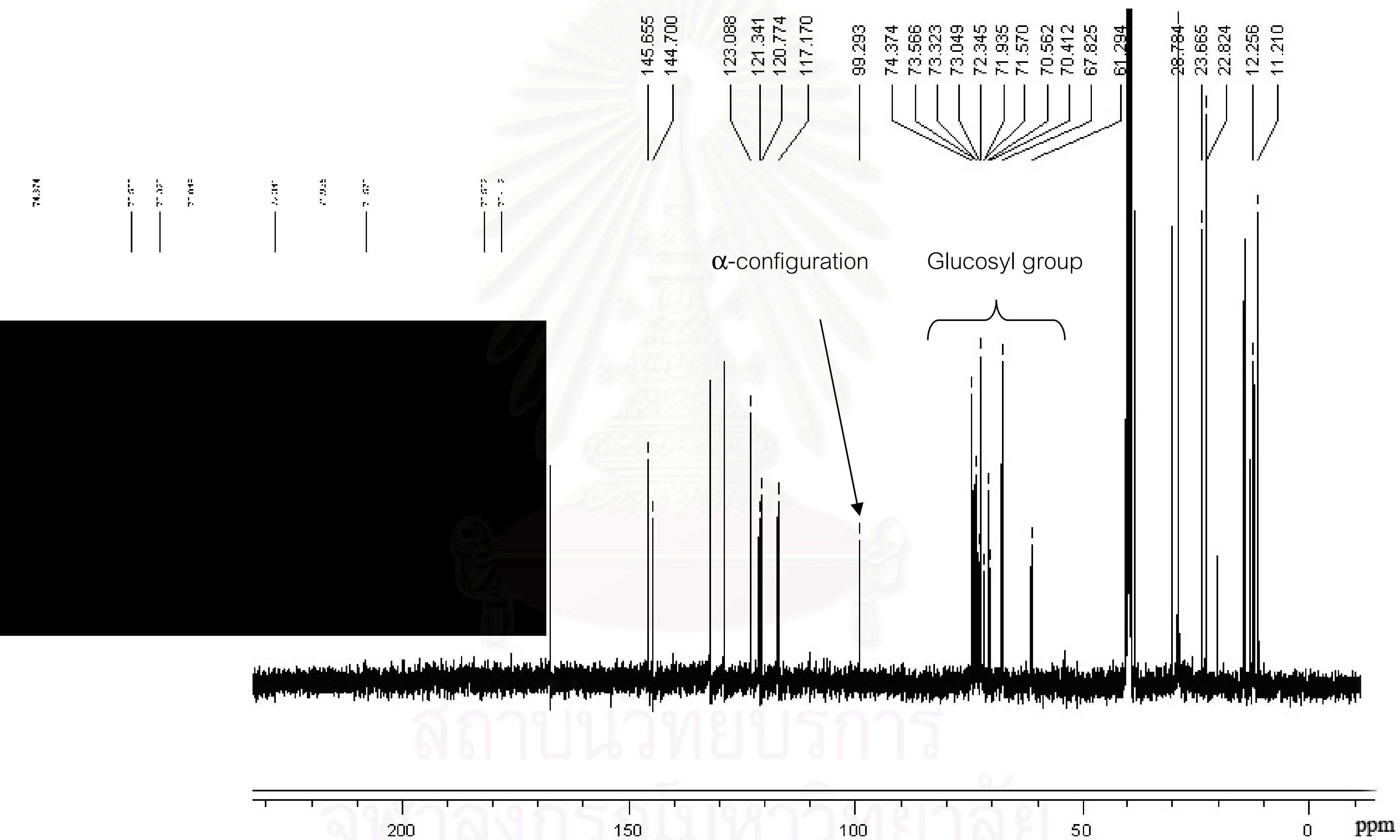

Figure 36. The 100 MHz ^{13}C-NMR of TMG. The inset indicated the spectra of the glucosyl group of TMG.

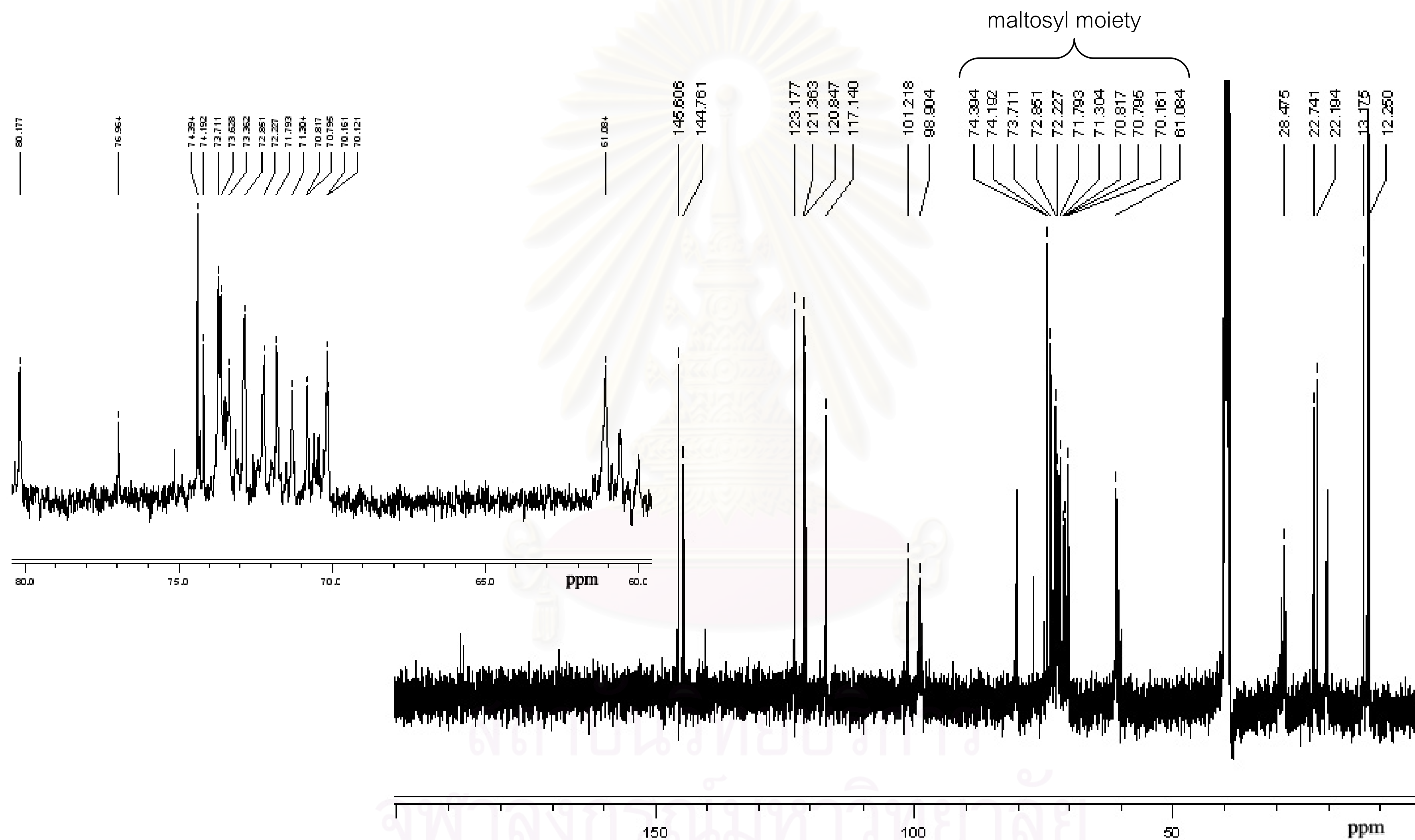

Figure 37. The 100 MHz ^{13}C-NMR of TMG_2. The inset indicated the spectra of the maltosyl moiety of TMG_2.

Table 8 ^{13}C NMR chemical shifts of TMG_2, TMG and TM from this experiment and (R,S)-TMG from Murase *et al.* report (1997)

TMG_2	TMG	TM	(R,S)-TMG from Murase *et al.* (1997)	Chromanol and glucose moiety
				Chromanol
74.4	74.4	75.3	73.8 and 73.9	2
70.2	67.8	67.2	70.0 and 70.1	2a
22.7	23.7	22.1	22.2 and 22.4	2b
28.5	28.8	28.4	28.2	3
22.2	22.8	20.4	19.7 and 19.8	4
117.1	117.2	117.3	116.65 and 116.72	4a
120.8	120.7	120.7	120.81 and 120.84	5
12.3	11.2	12.3	11.7	5a
144.8	144.7	145.0	144.2	6
121.4	121.3	121.4	120.17 and 120.21	7
13.2	12.2	13.2	12.6	7a
123.2	123.1	123.0	122.5	8
145.6	145.6	145.6	145.1	8a
12.3	11.2	12.3	11.7	8b
				Glucose
98.9	99.3		98.8	1’
72.9	73.0		72.6 and 72.9	2’
73.7	73.3		73.1	3’
76.9	71.6		71.2 and 71. 5	4’
71.7	72.3		71.9	5’
61.1	61.3		60.6 and 60.8	6’

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Table 8 (cont.) ^{13}C NMR chemical shifts of TMG_2, TMG and TM from this experiment and (R,S)-TMG from Murase *et al.* report (1997)

TMG_2	TMG	TM	(R,S)-TMG from Murase *et al.* (1997)	Chromanol and glucose moiety
				glucose
101.2				1''
72.2				2''
73.6				3''
71.3				4''
70.8				5''
61.1				6''

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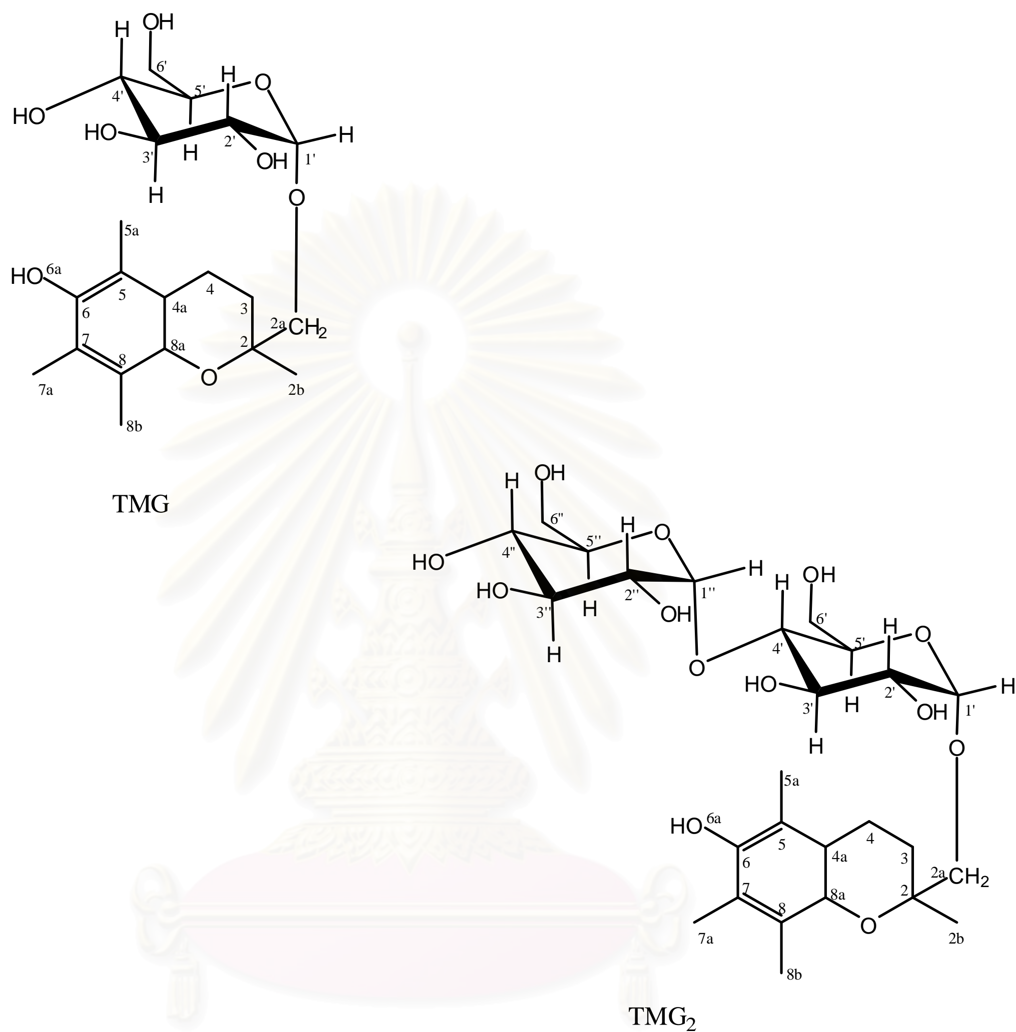

Figure 38. The molecular structure of 2-(α-D-glucopyranosyl)methyl-2,5,7,8-tetramethylchroman-6-ol (TMG) and α-D-glucopyranosyl-(1à4)-2-(α-D-glucopyranosyl)methyl-2,5,7,8-tetramethylchroman-6-ol (TMG_2).

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3.9 Fundamental properties of TMG and TMG_2

3.9.1 Solubility of TMG and TMG_2

The solubility of the novel vitamin E glycoside (TMG_2) was compared with those of TMG, TM and Trolox. The standard curve of each vitamin E analogue was plotted in mg per mL after HPLC analysis (Appendix 6). The solubility of TMG_2 was 1.08×10^4 mg/mL whereas the solubility of Trolox, TM and TMG were 0.149, 0.926 and 7.94×10^2 mg/mL, respectively. In other words, the solubility of TMG_2 was 14 times higher than that of TMG and was about 1×10^4 and 7×10^4 times higher than those of TM and Trolox, respectively. The results clearly showed that the attachment of glucosyl and maltosyl residues to TM by only α-glucosidase and by a two-step enzymatic reaction system (a combination of α-glucosidase and CGTase) expanded the water solubility over the original compound.

3.9.2 Scavenging activity of TMG and TMG_2

The free-radical scavenging activity of vitamin E analogues was determined by the use of the stable free-radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH). The DPPH radical is scavenged by the electron donated from the antioxidant, resulting in the decolorization and decrease in absorbance. After the absorbance at 516 nm was measured, the DPPH scavenging actvity (%) was calculated as described in Section 2.18. As shown in Table 9, the scavenging activity of TMG_2 was higher than those of TMG and TM and was nearly the same as that of Trolox.

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Table 9 The DPPH scavenging activity (%) of Trolox, TM, TMG and TMG_2 at the concentration of 0-200 μM.

Vitamin E analogue	Scavenging activity (%)					
	Concentration (μM)					
	0	10	25	50	100	200
Trolox	0	59.50	70.36	84.14	97.89	98.09
TM	0	54.07	58.57	62.17	72.63	90.41
TMG	0	56.23	67.07	76.01	89.26	95.71
TMG_2	0	58.68	69.97	80.00	95.58	95.85

The DPPH scavenging activity was calculated from DPPH assay. DPPH assay was performed by using various concentration of vitamin E analogue in ethanol/water (1:1 v/v) added to 0.25 mL of 0.5 mM DPPH in ethanol. After incubation at room temperature for 20 minutes, the decolorized DPPH was measured at 516 nm.

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CHAPTER IV

DISCUSSION

In this research, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as a vitamin E representative substrate to study the transglycosylation reaction by enzymatic method. Trolox is an analogue of vitamin E, the long phytyl side chain of which is substituted into the carboxylic moiety (Figure 8) to improve its solubility in water. In addition, the purpose for the production of Trolox was to substitute α-tocopherol (a major constituent of lipid-soluble vitamin E) for prevention from free radical oxidation in oil-in-water emulsions because the mobility was increased from shorter side-chain (Huang *et al.*, 1996). Moreover, Trolox is used as a standard in TEAC assay in order to determine the antioxidant capacity in food (Madhujith and Shahidi, 2005, Long *et al.*, 2000 and Pellegrini *et al.*, 2003). However, Trolox is still nearly insoluble in aqueous solution (0.16 mg/mL in water). Trolox was then modified by α-glucosidase-catalyzed transglucosylation using maltose as a donor to introduce glucose moiety into the molecule. Tocopherol monoglucoside (TMG), the glucoside product from this modification, becomes an excellent water-soluble antioxidant (Murase *et al.*, 1997). TMG was also found to have the radioprotective effect on hematopoietic system in mice. TMG was tested with mice and could prevent about 90% of bone marrow cells from the chromosome aberrations (Satyamitra *et al.*, 2001).

Transglycosylation is catalyzed by many kinds of enzymes so as to produce the glycosyl derivatives. The chemical properties such as solubility and/or stability of some compounds can be improved while still retaining their bioavailability. Cyclodextrin glycosyltransferase (CGTase) is one of the enzymes that can catalyze the transglycosylation reaction from a glycosyl donor to an acceptor and has widely been utilized for the purpose of developing many modified compounds. For examples, L-ascorbic acid with glucosyl moiety showed enhanced stability against oxidative

degradation in aqueous solution after sugar was added to L-ascorbic acid by free and immobilized CGTase (Tanaka *et al.*, 1991 and Prousoontorn *et al.*, 2007). The solubility of genistin, the isoflavone in soybean, was improved by transglycosylase activity of CGTase using starch as a donor. The solubility of maltosyl-α-genistin and glucosyl-α-genistin were about 3,700 and 40,000 times higher than that of genistin (Li *et al.*, 2005) and the bitterness of ginseng saponins were reduced by CGTase using dextrin as a donor (Kim *et al.*,2001). Thus, it is of great potential interest to synthesize vitamin E glycoside derivative in order to increase its solubility and still maintain the antioxidant activities.

4.1 TM synthesis

The carboxylic group of Trolox had to be reduced to the corresponding alcohol, 2-hydroxyethyl-2,5,7,8-tetramethylchroman-6-ol (TM) before the transglycosylation reaction catalyzed by CGTase could proceed.

TM was produced by strong chemical reduction as described by Murase *et al.* (1997). $LiAlH_4$ was used to reduce the carboxylic functional group of Trolox into hydroxyl group. However, the most important caution of this reaction was the incubation on ice and $LiAlH_4$ had to be gradually added otherwise the explosion could occur. Dried THF was used instead of diethyl ether, because THF had higher boiling point. Yield in mole percent of TM from this experiment (92.4%) was found to be almost equal to that of Murase *et al.* (1997) (95.3%). Moreover, ^{1}H-NMR spectra of TM from both experiments were identical. Therefore, it was believed that this compound was TM. Figure 39 shows the reaction how TM was synthesized by chemical method.

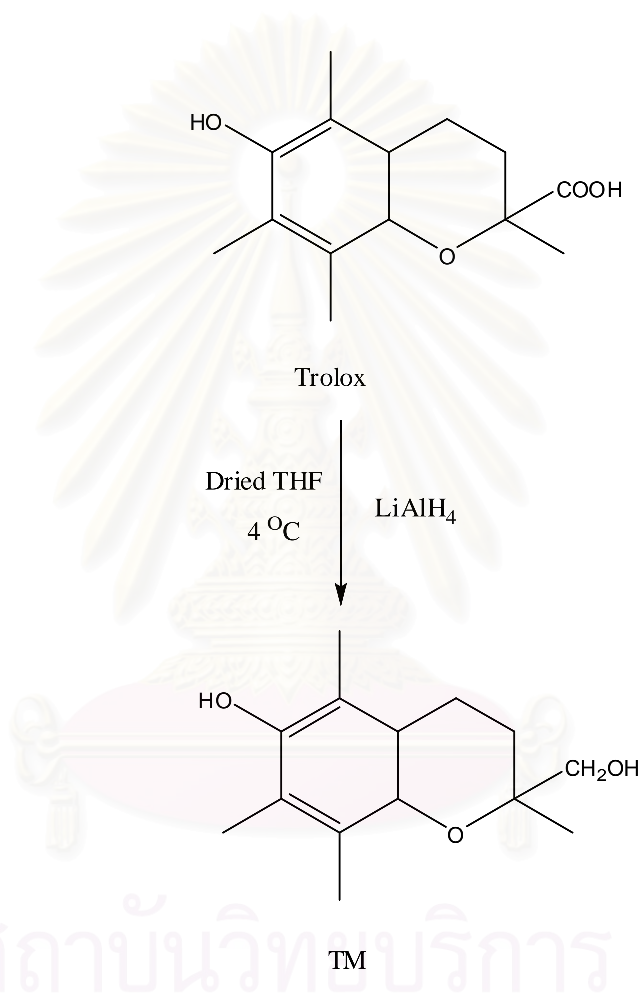

Figure 39. Schematic representation of the TM production using reducing property of $LiAlH_4$.

4.2 Transglycosylation to TM and TMG

In order to produce high amount of the water-soluble derivative of vitamin E for the practical application, many researches on vitamin E analogue synthesis have been carried out. Vitamin E tocopherol monoglucoside (TMG), which has a glucose residue conjugated to it, is one of these. TMG has been synthesized from TM and maltose by α-glucosidase (Murase *et al.*, 1997). The advantage of TMG over Trolox and α-tocopherol is its solubility which is extremely high. TMG can also prevent gastric mucosal damage, and micronuclei and chromosomal aberration in bone marrow from γ-irradiation without toxicity in rat (Ichikawa *et al.*, 2003 and Satyamitra *et al.*, 2003). Among various enzymes, CGTase seems to be effective in terms of the reaction specificity and efficiency. Therefore, it is of our great interest in tocopherol monoglucoside synthesis via transglycosylation reaction by CGTase. Unfortunately, TMG could not be synthesized. No product was found by TLC and HPLC analysis under standard conditions described in Materials and Methods, although the different kinds of donor were varied for transglycosylation. This could be due to the fact that TM was not a good acceptor for CGTase to catalyze the transglycosylation from glycosyl donor directly to TM. This has previously been observed with *l*-menthol (Do, 2002). Therefore, the production of vitamin E glycoside was modified into two step enzymatic reaction system: a combination of α-glucosidase and CGTase reactions (Figure 40).

Transglycosylation to TM was then performed according to the method previously reported by Murase *et al.* (1997). Then, this mixture was further incubated with CGTase and β-cyclodextrin as a glycosyl donor. For the detection of products, the reaction mixture was subjected to TLC and HPLC analysis. For TLC analysis by silica gel plates, the separation is based on their polarities, therefore, the compound with more polarity will move slower and vice versa. It can clearly be seen that the spots of TM, TMG and TMG_n were separated corresponding to their polarities with the R_f values of 0.94, 0.46 and 0.22, respectively (Figure 21A). The HPLC analysis was performed on a reverse phase C_{18} column. The separation of the compounds is also based on their polarities. The stationary phase comprised of octadecyl group (C_{18}) which is highly non-

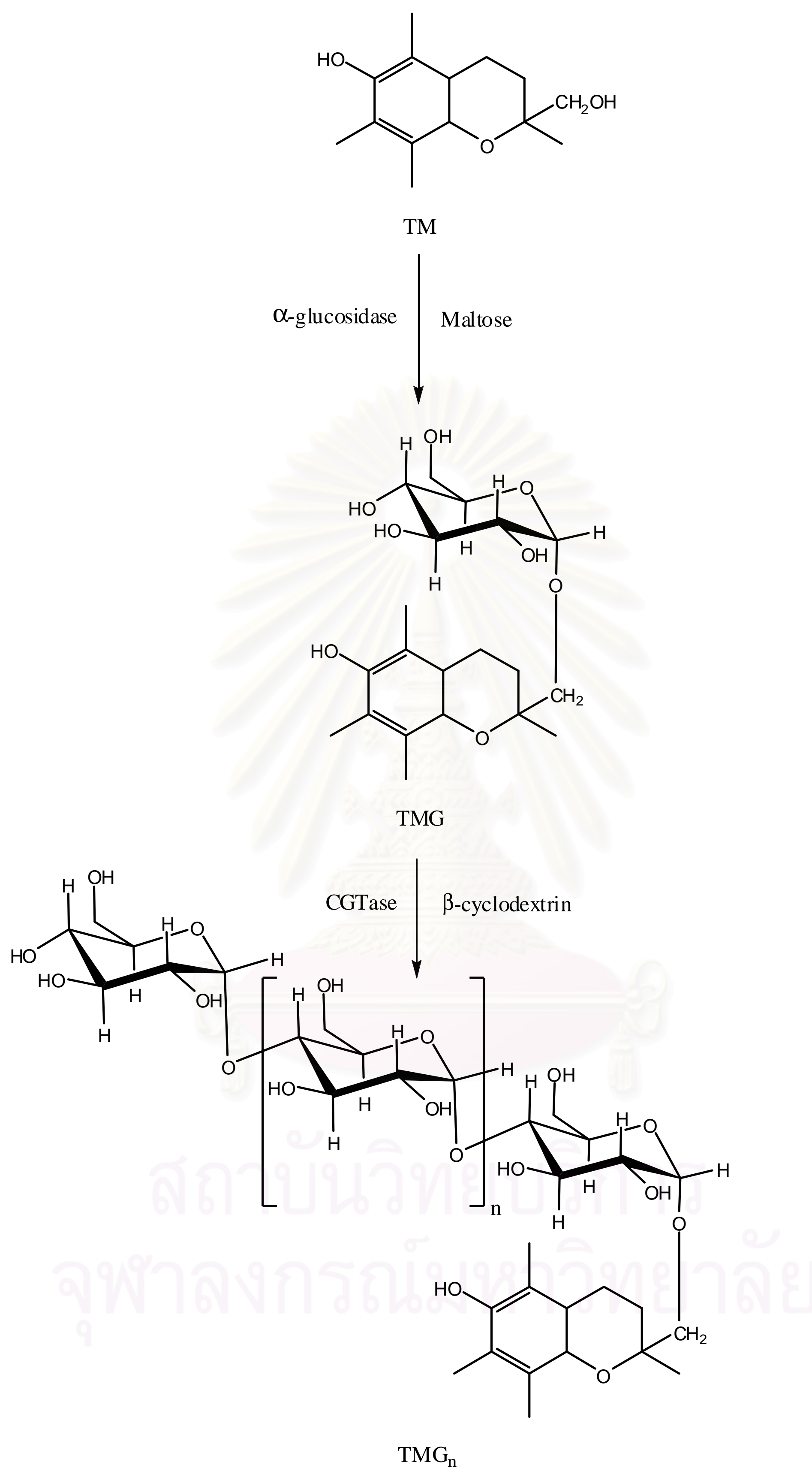

Figure 40. Schematic representation of TMG_n production by α-glucosidase and CGTase.

polar. Thus, the compound with less polarity was eluted with higher retention time. As shown in HPLC chromatogram (Figure 21B), the TMG_n was eluted before TMG and TMG was eluted before TM. From these results, it is quite obvious that TMG_n was produced by CGTase when TM was glucosylated in the first step by α-glucosidase and CGTase could then elongate the α-glycosidic chain of TMG in the second step (Figure 40). To confirm that the product was produced by the action of CGTase that catalyze the transglycosylation to TMG from β-CD, glucoamylase was introduced to catalyze the hydrolysis of glycosidic linkages between glucose residues in the reaction mixture. After HPLC analysis, it was found that the peak of TMG_n disappeared with increasing amount of TMG (Figure 24). Therefore, it was presumed that the novel compound was TMG_n composing of glucose residue at least one molecule attached to TMG.

4.3 Optimization of TMG_n production

In previous study, Murase *et al.* (1997) demonstrated the successful production of TMG by α-glucosidase. Therefore, TMG was synthesized according to their procedure. After the TMG was synthesized, we carried out the glucose transfer reaction with CGTase using TMG and β-CD as a glycosyl acceptor and donor, respectively. Factors such as the concentration of donor, acceptor, enzyme and the reaction pH, temperature and reaction time which influence the production of TMG_n were then investigated.

First, the reaction mixture was incubated at various temperatures ranging from 30 to 70 ^{O}C. Since this CGTase was obtained from thermotolerant *Paenibacillus* sp.RB01 and was found to be most stable at 45-55 ^{O}C (Yenpetch, 2002). It was found that the range of the 30-50°C can be used for transglycosylation with optimum temperature at 50°C (Figure 25). At temperature higher than 50°C, the amount of TMG_n formed was lower which could be explained by low enzyme activity due to enzyme inactivation.

Next, three kinds of buffer, acetate, phosphate and Tris-glycine buffer were used to vary pH of the reaction mixture. Although the phosphate buffer at pH 6.0 gave the best result, it was discovered that at pH 9 and 10, the amount of TMG_n was comparable

to that of pH 6 (Figure 26). From the previous report, CGTase from *Paenibacillus* sp. RB01 is an alkalophilic enzyme, which is stable over pH range 6.0-10.0 (Yenpetch, 2002). Therefore, the range of pH for transglycosylation is also broad.

Time course on TMG_n synthesis was performed during 48 hours. For every 4-hour-detection, it was found that the time used for the production of TMG_n was surprisingly short (4 hours) (Figure 27). This could be resulted from a glucosyl moiety on TMG molecule which acts as an appropriate acceptor for CGTase to rapidly catalyze the transglycosylation reaction. After 4 hours, the amount of TMG_n slightly decreased. Even though, the frequency of the detection was increased into the 2-hour-detection, the appropriate time for incubation was still at 4 hours (data not shown).

For the determination of the effect of donor, acceptor and enzyme concentration on the TMG_n production, TMG needed to be purified. TMG production was performed according to the method described by Murase *et al.* (1997). The reaction mixture was concentrated by evaporator and TMG was purified by Amberlite XAD-4, the polystyrene resin used for DMSO and sugar removal. TMG was checked by HPLC and the purification profile was drawn by 280 nm spectrophotometer detection (Figure 29). TMG could be separated from TM by this method; however, the flow rate of the mobile phase was limited (not more than 0.5 mL/min). Methanol at 30% (v/v) was used to elute DMSO and sugar before 80% (v/v) of methanol was used to elute TM and TMG. The mole percent yield of production in large scale (37.85%) was higher than that of Murase *et al.* (1997) report (14.84%). The higher yield of TMG in this experiment was possibly resulted from the purification in one step. This was different from the Murase *et al.* report (1997) in that TMG was further purified by silica gel column chromatography to separate the denatured α-glucosidase.

The optimized CGTase and substrate concentration was obtained after HPLC analysis was performed. The unit of enzyme was calculated by dextrinizing activity. Although the amount of CGTase at 150 U gave the highest production of TMG_n, the production yield (%) was lower than that of 100 U of CGTase (Figure 30). Therefore, the

CGTase concentration of 100 U was further used. These cost high degree of CGTase concentration comparing with the previous report of transglycosylation reaction of some compounds by CGTase such as, 100 U of CGTase from *Bacillus stearothermophilus* for glycosyl glycerol production (Nakano *et al.*, 2001), 200 U of CGTase from *Bacillus macerans* for curcumin β-maltooligosaccharides production (Shimoda *et al.*, 2007). For the β-CD concentration optimization, the amount of TMG_n was maximum at a final concentration of 1.5% (w/v) β-CD (Figure 31). Moreover, β-CD at concentration higher than 1.8% (w/v) has low solubility in water at 25 ^{O}C. Thus, when β-CD at concentration higher than 1.8% was used, the solution became turbid during incubation. For these reason, the final concentration of 1.5% (w/v) β-CD was further used. Next, the amount of TMG used was optimized. The TMG concentrations were varied to find the optimum TMG supplied. Although the amount of TMG_n increased with increasing concentration of TMG, the production yield was not significantly different and the final TMG concentration of 0.5% (w/v), which had the highest production yield, was then used for the large-scale production of TMG (Figure 32). The concentrations of the donor and acceptor used in transglycosylation reaction by CGTase that have been reported are generally varied depending on the type of donor and acceptor used. Tanaka *et al.* (1971) reported the use of 12.8% (w/v) of α-CD as donor in the transglycosylation to ascorbic acid (7.04% w/v) by CGTase. The production of *l*-menthol with a maltosyl group attached was investigated in the reaction mixture containing 0.3% (w/v) soluble starch and 1.5% (w/v) *l*-menthyl glucoside (Do *et al.*, 2002). For the formation of hesperidin glycoside, hesperidin as an acceptor at 0.1% (w/v) and soluble starch as a glycosyl donor at 5% (w/v) were used as the standard conditions (Kometani *et al.*, 1994).

4.4 Large-scale TMG_n production

TMG_n production under optimal conditions (0.5% (w/v) of TMG, 1.5% (w/v) of β-CD, 100 U of CGTase, pH 6.0, 50 ^{O}C, 4 hours) was scaled up 60 times. After the reaction mixture was incubated for 4 hours and concentrated, it was passed through the silica gel column chromatography. The flow rate of mobile phase should be slow to

increase the efficiency. However, HPLC peak still showed some impurities at R_t of 4.9, 9.5 and 10 minutes. It might be an effect from solvent (ethyl acetate, methanol or water) which was detected before it was removed. The mole percent yield of TMG_n production (17.57%) was similar to other glycosyl compounds synthesizing by CGTase such as 16.10% of *l*-menthol α-maltoside production (Do *et al.*, 2002), 14.50% of glycerol glucoside production (Nakano *et al.*, 2003) and 12.40% 2-*O*-α-D-glucopyranosyl L-ascorbic acid production (Bae *et al.*, 2001).

4.5 Molecular weight and structure of TMG and TMG_n

Molecular weight of TMG and TMG_n were identified by ESI-TOF MS in the positive mode $[M+Na]^+$. In electrospray ionization (ESI) part, the TMG and TMG_n was dissolved in 50% (v/v) methanol and the quasimolecular ions of TMG and TMG_n was created by addition of sodium $[M+Na]^+$. Then, the ions would pass through one-meter tube as a mass analyzer of time of flight (TOF) part and detected by time array detector. The value of m/z from ESI-TOF MS in the positive mode composes of molecular weight of sodium (at m/z 23) plus the molecular weight of TMG and TMG_n. TMG molecule showed three signals which have the similar molecular weight (at m/z 409.1648, 421.1860 and 437.1883). Fortunately, the molecular weight of TMG was previously reported at m/z 398 by Murase *et al.* (1997). Therefore, the molecular weight at m/z 421.1860 should be TMG (MW = 398.1960), which was comparable with the calculation by MS (421.1833) (Figure 34). Other peaks might be caused by the interference of sodium interaction. While TMG needed to have reference for identification, TMG_n was easier to identify. The molecular mass of TMG_n from ESI-TOF and calculation were nearly the same (at m/z value 583.2363 and 583.2361, respectively). The molecular weight of TMG_n was calculated by the subtraction of the molecular mass from ESI-TOF by that of sodium (at m/z 583.2363 – 23 equals 560.2363). When the molecular weight of TMG_n was subtracted by that of TMG, the value would be 162.0503, which was similar to the molecular weight of one glucosyl residue ($C_6H_{12}O_5$ equals 164.0685 from

calculation). It is possible to say that TMG was transglucosylated by CGTase. The structural confirmation by NMR was further done.

The molecular structure of TMG and TMG_n was further identified by ^{13}C-NMR (400 MHz). It can be seen that that the ^{13}C NMR spectra of TMG from our experiment were similar to those of TMG from Murase *et al*. report (1997) (Figure 36 / Table 8). TMG was then identified as 2-(α-D-glucopyranosyl)methyl-2,5,7,8-tetramethylchroman-6-ol. For TMG_n, chemical shifts were compared to those of TMG from this experiment in ^{13}C NMR spectra. As shown in Figure 37 and Table 8, additional carbon signals were observed. Unquestionably, those signals resulted from the transfer of glucose moiety to TMG. In addition, there was a large chemical shift of C-4' in glucose moiety of TMG from 71.6 to 76.9, confirming that the transferred glucosyl was attached to C-4' in glucose moiety of TMG. These results were similar to ^{13}C NMR spectra of l-menthyl maltoside , which had α-maltosyl group (Do *et al*., 2002). This concluded the TMG_n had an α(1à4)-glycosidic linkage between glucose units combining these results together, the transglycosylation product by CGTase was elucidated as α-D-glucopyranosyl)-α(1à4)-2-(α-D-glucopyranosyl)methyl-2,5,7,8-tetramethylchroman-6-ol (TMG_2).

4.6 Some properties of TMG and TMG_2

4.6.1 Solubility of TMG and TMG_2

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Distilled water was used to dissolve the entire vitamin E analogues. Trolox had the most difficulty to be dissolved in water due to its low solubility. TMG_2 in this experiment could be dissolved in water in highest amount comparing to other vitamin E analogues. Its solubility was 14 times higher than that of TMG and was more than 1×10^4 and 7×10^4 times higher than those of TM and Trolox, respectively. This resulted from the introduction of 2 glucose residues into the molecule of vitamin E. Thus, the solubility of the TMG_2 was found to be even higher than that of TMG. This was in a good agreement with other previous reports. For examples, the solubility of glucosyl-α(1à4)genistin and maltosyl-α(1à4)genistin was 3.7×10^3 and 4.4×10^4 times higher

than that of genistin (Li *et al.*, 2005) and the solubility of *l*-menthyl α-maltoside was 1.6 x 10^3 times higher than that of *l*-menthyl α-glucoside (Do *et al.*, 2002). However, the solubility of TMG from our experiment (793.8 mg/mL) was slightly lower than that from previous report (more than 1,000 mg/mL of TMG, Murase *et al.*, 1997). This might result from the impurities remained in TMG, which were not eliminated after only one step purification by Amberlite XAD-4. Nevertheless, the solubility of TMG was also 1 x 10^3 and more than 6 x 10^3 higher than those of TM (0.926 mg/mL) and Trolox (0.149 mg/mL) and this corresponded to Murase *et al.* (1997) report.

4.6.2 Scavenging activity of TMG and TMG_2

The antioxidant activity of transglycosylated products was examined by exposure to oxidation conditions. Generally, free radical scavenging activity can be used to evaluate an antioxidant effect of certain chemicals. Therefore, the scavenging activity (%) of TMG and TMG_2 was determined by bioassay for the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). However, DPPH method was slightly modified in this experiment because the low solubility in water of Trolox. Trolox needed to be dissolved in higher concentration of ethanol to reach the same concentration as other vitamin E analogues. As can be seen in Table 9, the scavenging activity (%) of TMG and TMG_2 were similar to that of Trolox and thus, TMG_2 and TMG can prevent the free-radical in the aqueous system with the same antioxidant power as Trolox in the lipid-soluble system. In conclusion, the attachment of maltosyl group to vitamin E at the –OH position at the side chain did not effect of the antioxidant activity of the vitamin E.

TMG_2, the novel vitamin E glycoside, was synthesized by a two-step enzymatic transglycosylation system consisting of a combination of α-glucosidase from *Saccharomyces cerevisiae* and CGTase from *Paenibacillus* sp. RB01. The scavenging activity of water-soluble TMG_2 by DPPH method was nearly the same as TMG in water and Trolox in organic solvent. TMG_2 can be applied in the industry such as drug and supplementary food to help people who have difficulty absorbing fat (cystic fibrosis in digestive system) and increase the vitamin E absorption at intestinal wall. The water-

soluble vitamin E formulation (Aqua-E) was compared with oil-based softgels in pharmacokinetic study. It was found that the absorption of total tocopherols in Aqua-E showed 3 times higher than that of oil-based softgels (Papas *et al.*, 2007). Therefore, this water-soluble vitamin E, TMG_2, is possibly used as ingredient in the production of drug and supplementary food for vitamin E malabsorbing patient.

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CHAPTER V

CONCLUSIONS

1. CGTase from thermotolerant *Paenibacillus* sp. RB01 was partially purified by starch adsorption. The specific activity was 1825.7 U/mg with the purification fold of 17.4 and the obtained yield was 83%.
2. TMG_2 was successfully produced by a two-step sequence of transglycosylation by α-glucosidase and CGTase.
3. The optimum conditions for CGTase transglycosylation were to incubate TMG at a final concentration of 0.5% (w/v) and 1.5% (w/v) of β-cyclodextrin with 100 U of CGTase at pH 6.0 for 4 hours at 50 ^{O}C.
4. Under optimized conditions, the transglycosylation yield of TMG_2 was calculated to be 47.2 % from peak area.
5. After large-scale production of TMG_2 was performed and purified by silica gel column chromatography, yield of transglycosylation of TMG_2 was 17.6 in mole percent.
6. Molecular weight of TMG and TMG_2 from ESI-TOF MS were 398 and 560, respectively. TMG was identified as 2-(α-D-glucopyranosyl)methyl-2,5,7,8-tetramethylchroman-6-ol and TMG_2 was identified as α-D-glucopyranosyl-α(1→4)-2-(α-D-glucopyranosyl)methyl-2,5,7,8-tetramethylchroman-6-ol.
7. The solubility of TMG_2 was 14 times higher than that of TMG and was more than 1×10^4 and 7×10^4 times higher than those of TM and Trolox, respectively.
8. The free-radical scavenging activity of TMG and TMG_2 against a stable free-radical, DPPH, exhibited nearly the same degree as that of Trolox.

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APPENDICES

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APPENDICES

Appendix 1: Preparation for SDS-Polyacylamide gel electrophoresis

- ### Stock reagent

2 M Tris-HCl, pH 8.8

Tris(hydroxylmethyl)-aminomethane	24.2	g

Adjusted pH to 8.8 with 1 M HCl and adjust volume to 100 mL with distilled water

1 M Tris-HCl, pH 6.8

Tris(hydroxylmethyl)-aminomethane	12.1	g

Adjusted pH to 6.8 with 1 M HCl and adjust volume to 100 mL with distilled water

10 % (w/v) SDS

Sodium dodecyl sulfate	10	g

Adjusted volume to 100 mL with distilled water

50 % (v/v) glycerol

Glycerol	50	mL

Added 50 mL water and mix well

1 % (w/v) bromophenol blue

Bromophenol blue	100	mg

Added in 10 mL distilled water and mixed well. Then, solution was filtered to eliminate the aggregated dye.

- ### Working solutions

Solution A

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

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

Adjusted volume to 100 mL with distilled water

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Solution B

4x Separation Gel buffer

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

Solution C

4x Stacking Gel buffer

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

10 % ammonium persulfate

Ammonium persulfate	0.5	g
Distilled water	5	mL

Electrophoresis buffer

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

Adjust volume to 1 liter with distilled water

5x Sample Buffer

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

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Appendix 2: The composition of $KMnO_4$ solution for TLC detection

Potassium permanganate ($KMnO_4$)	3	g
Potassium carbonate	20	g
5% NaOH in water	5	mL

Adjust volume to 300 mL with distilled water

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Appendix 3: Preparation of buffer for TMG_2 synthesis

- 50 mM acetate buffer, pH 4, 5 and 6

100% acetic acid	0.4505 g
Sodium acetate trihydrate	1.0206 g

Adjusted pH to 4, 5 and 6 by 1 M acetic acid and adjusted volume to 150 mL

- 50 mM phosphate buffer, pH 6, 7, 8

di-Potassium hydroden phosphate	1.3064 g
Potassium dihydroden phosphate	1.0208 g

Adjusted pH to 4, 5 and 6 by 1 M acetic acid and adjusted volume to 150 mL

- 50 mM Tris-glycine buffer, pH 8, 9 and 10

Tris(hydroxylmethyl)-aminomethane	0.9085 g
Glycine	0.5631 g

Adjusted pH to 8, 9 and 10 by 1 M NaOH and adjusted volume to 150 mL

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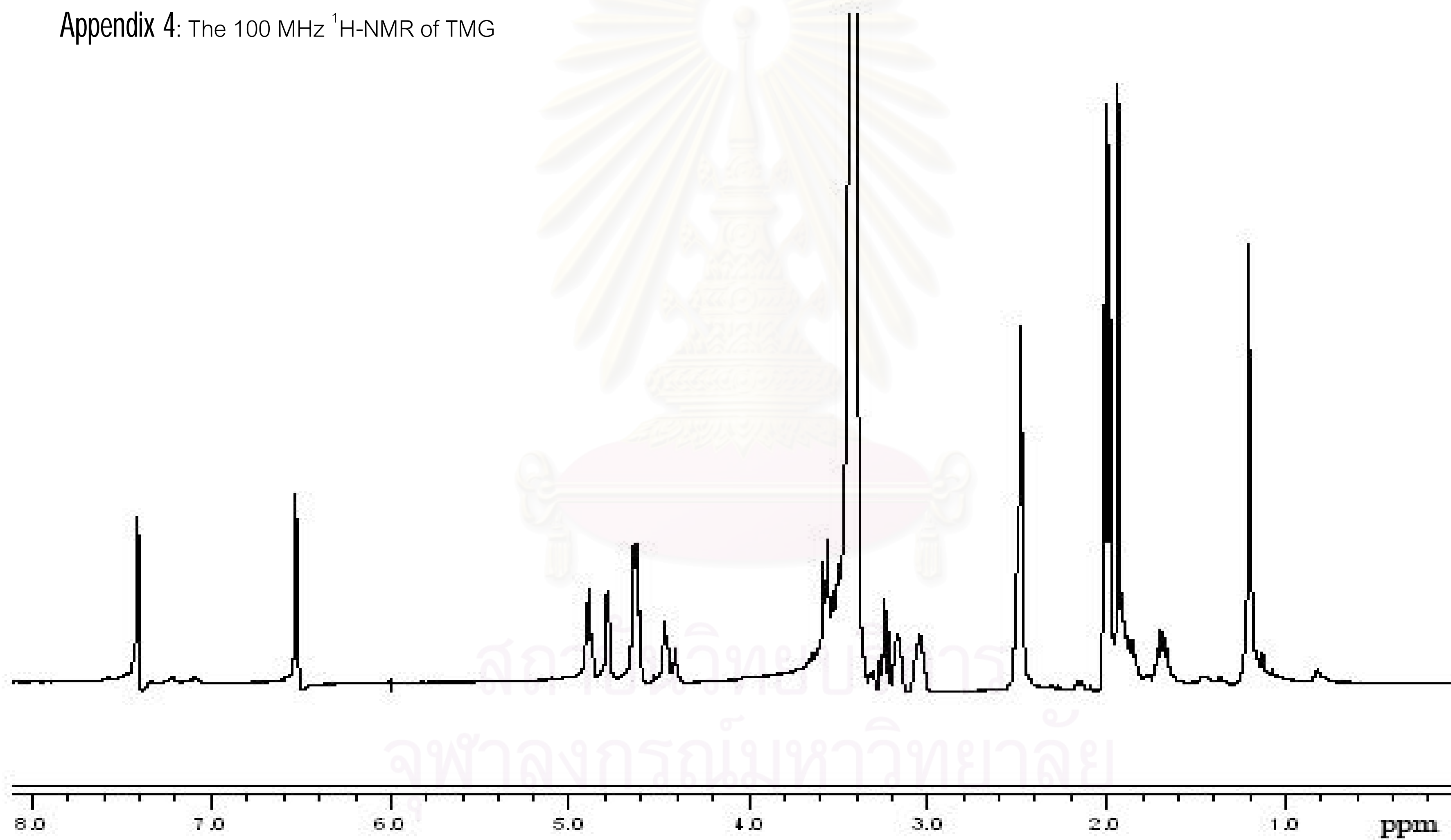
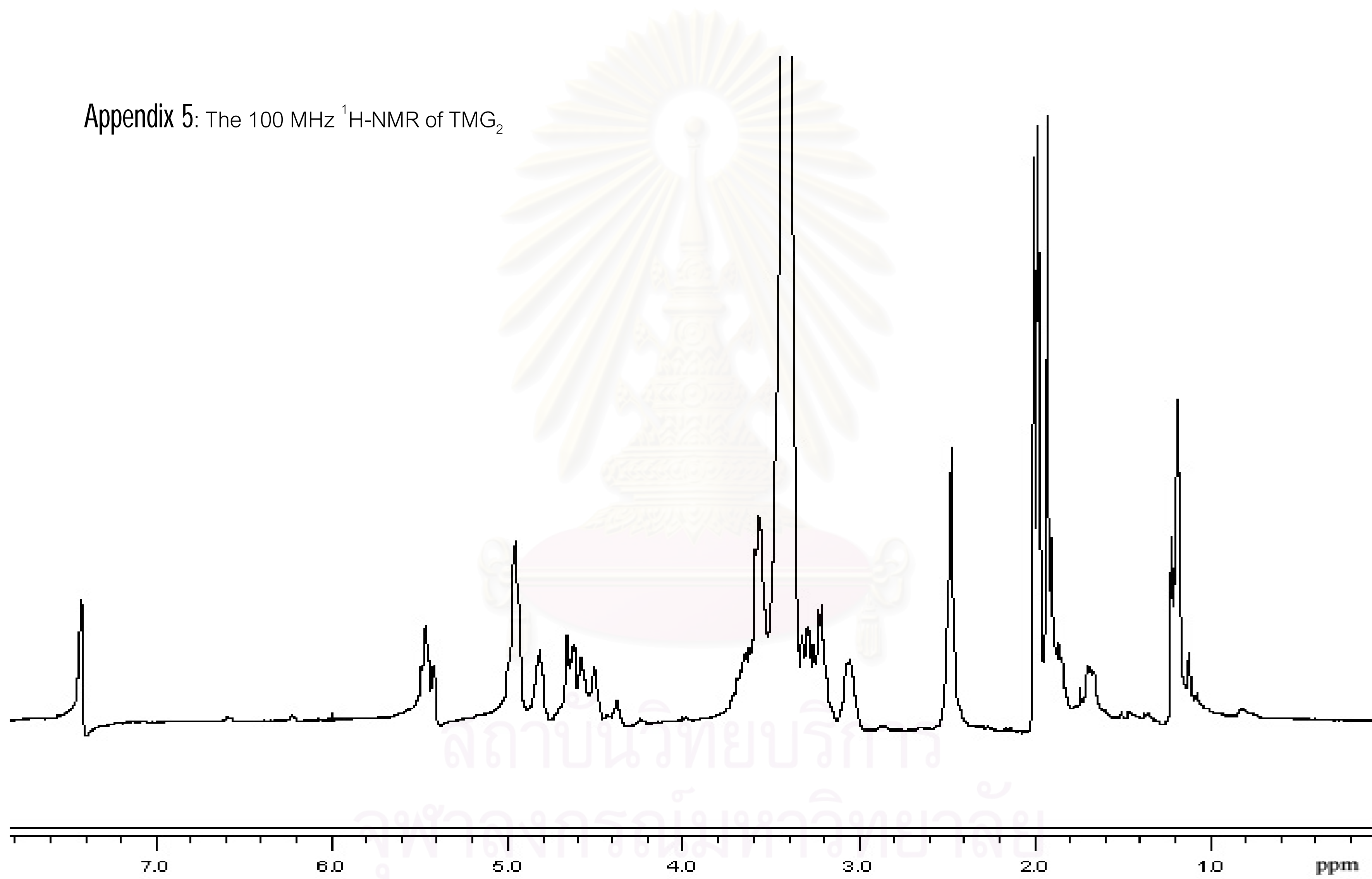
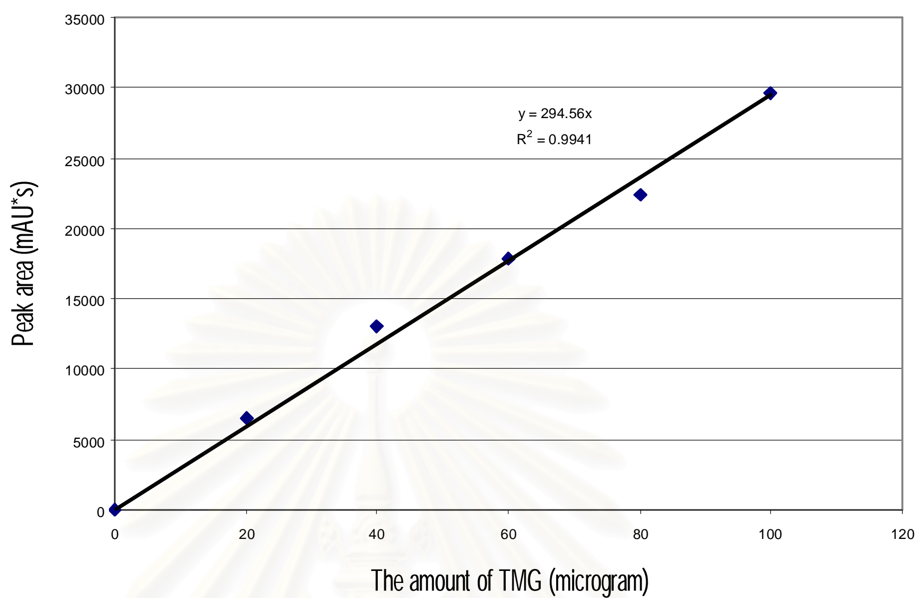
Appendix 4: The 100 MHz ^{1}H-NMR of TMG



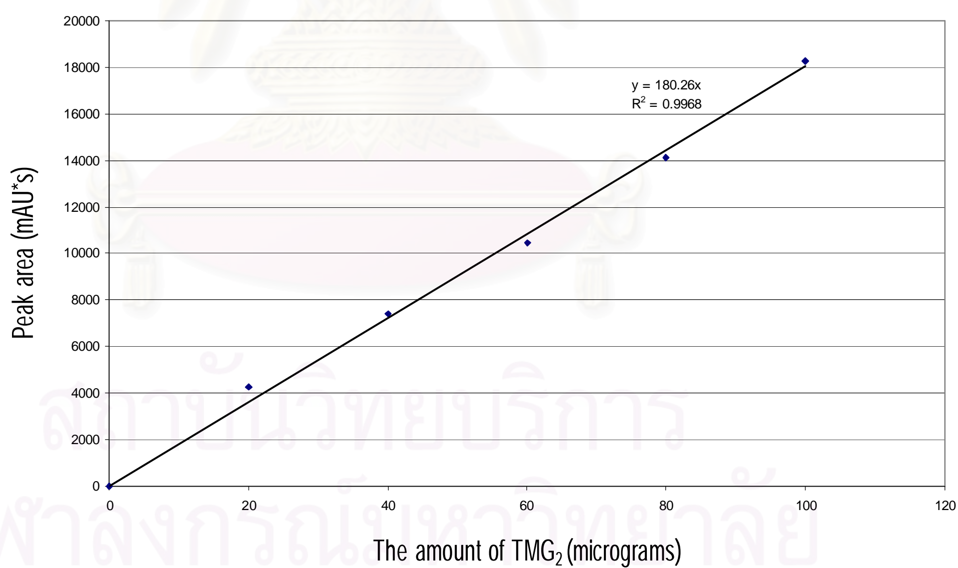
Appendix 5: The 100 MHz ^{1}H-NMR of TMG_2



Appendix 6: Standard curve of the solubility of vitamin E analogue

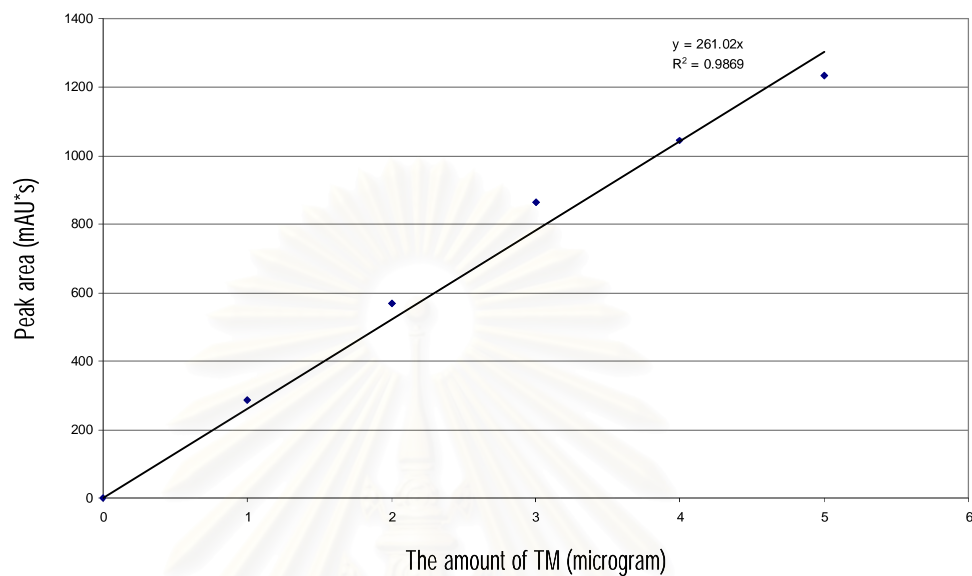


6.1 Standard curve of the solubility of TMG

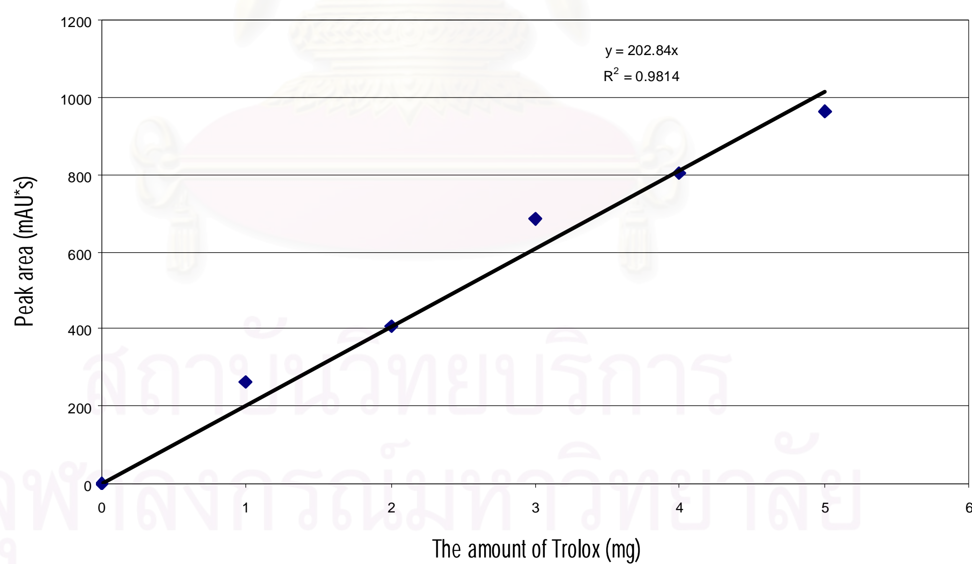


6.2 Standard curve of the solubility of TMG_2

Appendix 7 (cont.): Standard curve of the solubility of vitamin E analogue

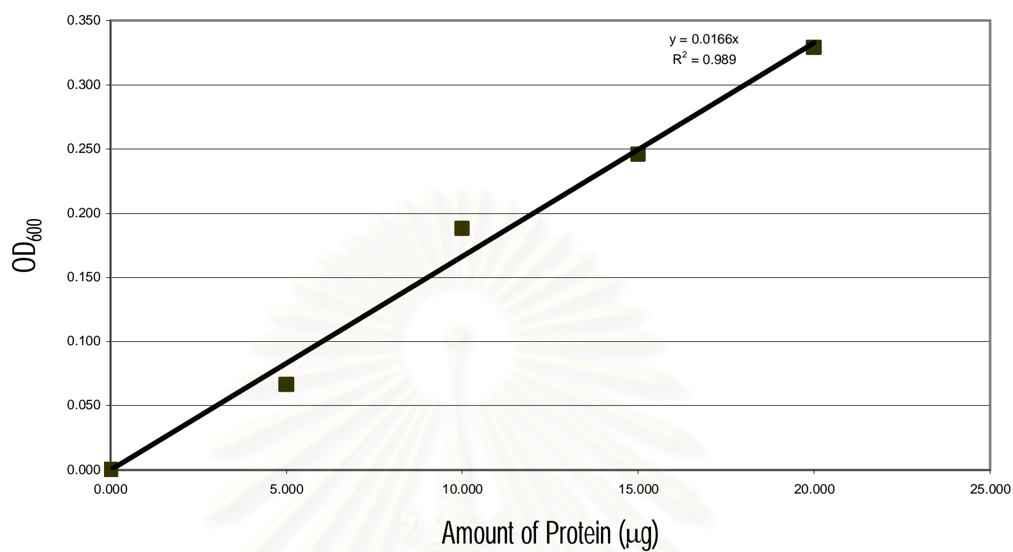


6.3 Standard curve of the solubility of TM



6.4 Standard curve of the solubility of Trolox

Appendix 8: Standard curve of the determination of protein by Bradford's method.



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BIOGRAPHY

Mr. Pisanu Thanadolsathien was born on May 20, 1982. He graduated from Bachelor Degree of Science in Biotechnology, Mahidol University in 2004. He has been studying in Master degree in the field of Biotechnology since June 2004.

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