รายงานวิจัยฉบับสมบูรณ์

# โครงการ การสกัดแยกสารยับยั้งเอนไซม์ย่อยคาร์โบไฮเดรต จากสมุนไพรไทย

# Screening and Isolation of Carbohydrate Digestive Enzymes Inhibitors from Thai Herbs

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## บทคัดย่อ

สารออกฤทธิ์ยับยั้งการทำงานของเอนไซม์อัลฟากลูโคซิเดสจะทำให้การย่อยสลายแป้งและโอลิโกแซคคาไรด์สเกิดขึ้นช้าลง ซึ่งส่งผลให้การดูดซับน้ำตาลเข้าสู่กระแสเลือดลดลงและนำไปสู่แนวทางการรักษาโรคเบาหวานชนิดไม่พึ่งพาอินซูลิน ดังนั้นจุดประสงค์ของโครงการวิจัยนี้คือการสกัดแยกสารออกฤทธิ์ที่สามารถยับยั้งเอนไซม์ย่อยคาร์โบไฮเดรตสองชนิดคือ ซูเครสซึ่งสกัดได้จากลำไส้เล็กของหนู และอัลฟาอมิลสที่สกัดมาจากกระเพาะหมู (PPA) จากสมุนไพรไทยหลายชนิด และเพื่อทำการศึกษาความเป็นไปได้ของสมุนไพรไทยในการยับยั้งการทำงานของเอนไซม์ดังกล่าวในมนุษย์โดยใช้การเพาะเลี้ยงเนื้อเยื่อของเซลล์มะเร็งชนิด Caco-2 เป็นแบบจำลองในการศึกษา

จากผลการสกัดพืชสมุนไพรไทยด้วยห้าสิบเปอร์เซ็นต์เมธานอลพบว่าพืชสมุนไพร 6 ชนิดจาก 47 ชนิดให้ค่าการยับยั้งการทำงานของเอนไซม์ซูเครส และ PPA ได้สูงทั้งนี้รวมไปถึง *Morus alba* Linn (Mulberry) หรือใบหม่อน ซึ่งพบว่าสามารถยับยั้งเอนไซม์ซูเครสได้ 85.70% และ PPA 94.71% ดังนั้นจึงนำส่วนที่ละลายได้ในชั้นของเอธิลอะซิเตทมาทำการแยกให้บริสุทธิ์โดยใช้คอลัมน์โครมาโตกราฟีและ HPLC จากนั้นทำการศึกษาโครงสร้างด้วย MS และ NMR พบว่าสารออกฤทธิ์ที่พบคือสารประกอบของกรดปาลมิติกและกรดโอลิอิก ซึ่งสามารถยับยั้งการทำงานของเอนไซม์ PPA ได้ 71% นอกจากนี้ยังพบว่าในชั้นน้ำของสารสกัดห้าสิบเปอร์เซ็นต์เมธานอลจากใบหม่อนให้ค่าการยับยั้งเอนไซม์ซูเครสและมอลเตสสูง ประกอบกับในปัจจุบัน mulberry หรือ ใบหม่อนได้รับความนิยมในการบริโภคในรูปของชาใบหม่อน ดังนั้นจึงทำการศึกษาผลของสารออกฤทธิ์ของชาใบหม่อนจาก 4 ผลิตภัณฑ์และระยะเวลาที่ใช้ในการชงชาที่ต่างกัน ผลการศึกษาพบว่าการยับยั้งเอนไซม์มีค่ามากที่สุดในเวลาการชงชาที่ต่างกันในแต่ละผลิตภัณฑ์แต่สามารถสรุปได้ว่าระยะเวลาในการชงชาตั้งแต่ 3-5 นาที สามารถให้ประสิทธิภาพในการยับยั้งการทำงานของเอนไซม์ได้สูงสุด ในการศึกษาโดยใช้แบบจำลองของเซลล์ Caco-2 พบว่าชาใบหม่อนสามารถลดปริมาณกลูโคสในชั้นอพิคัลและชั้นเบซัลของ Caco-2 monolayer ได้ ดังนั้นจึงสามารถสรุปได้ว่าชาใบหม่อนให้สารออกฤทธิ์ที่สามารถยับยั้งการทำงานของเอนไซม์ PPA, ซูเครส และมอลเตส และมีความเป็นไปได้ที่จะใช้บริโภคเป็นชาสมุนไพรสำหรับป้องกันโรคเบาหวาน แต่อย่างไรก็ตามปริมาณของสารออกฤทธิ์ในชาใบหม่อนควรจะต้องทำการศึกษาต่อไป นอกจากนี้ผลการศึกษาที่ได้จากโครงการวิจัยนี้พบว่านอกเหนือจากใบหม่อนแล้วยังมีสมุนไพรไทยอีกหลายชนิดที่มีศักยภาพในการยับยั้งการทำงานของเอนไซม์ย่อยคาร์โบไฮเดรต ดังนั้นควรจะมีการศึกษาสกัดแยกสารออกฤทธิ์ชนิดอื่นๆ จากสมุนไพรดังกล่าวในอนาคต

**คำหลัก** เอนไซม์อัลฟากลูโคซิเดส, *Morus alba* Linn. (Mulberry), ใบหม่อน, Caco-2

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## Abstract

Using inhibitors of α-glucosidase enzymes, which would delay the degradation of starch and oligosaccharides, therefore decrease the glucose absorption is a prospective approach for treating noninsulin-dependent diabetes mellitus. Therefore, the aim of this study was to isolate inhibitory active compounds against two α-carbohydrate digestive enzymes, rat intestinal sucrase and porcine pancreatic α-amylase (PPA), from various kinds of Thai herbs and to study the effect of such isolated active compounds on carbohydrate digestion using Caco-2 cell culture.

Thai herbs samples were extracted with 50% MeOH and found that 6 of the 47 tested plant materials showed high α-amylase and sucrase inhibitory activity including *Morus alba* Linn. (Mulberry) leaves (85.70% and 94.71%, respectively). The ethyl acetate partitioned fraction of mulberry extract, which showed high PPA inhibitory activity was basically purified using column chromatography and the eluted active fractions were further purified by HPLC. Chemical structure analysises by MS and NMR measurements revealed that the active constituents from mulberry leaves extract was a mixture of palmitic and oleic acids which showed 71% PPA inhibitory activity. In addition, sucrase and maltase inhibitory activities of the hot water extract of mulberry teas (Mon tea) purchased from different local markets of Thailand were compared. The results showed that although there were significant differences of product varieties in highest inhibitory activity against both sucrase and maltase, the brewing time of 3 to 5 min in tea preparation showed the most effective enzyme inhibition. In a Caco-2 cell culture experiment the investigated mulberry tea reduced the liberated glucose contents in both apical and basal sides of the cell monolayers. It can be concluded that hot water extract of mulberry leaves, (mulberry tea) does have inhibitory effect against α-glucosidases, PPA, sucrase and maltase enzymes, and has potential possibility to be consumed as antidiabetic herb tea. We propose that the quantitative measurement of active compounds in hot water extract of mulberry tea infusion should be performed. In addition, the other prospective samples showed high enzyme inhibitiory acitivities found in this study should be investigated further.

**Keywords:** α-glucosidases inhibitors, *Morus alba* Linn. (Mulberry), Caco-2 cell culture

# สารบัญ

หน้า

# Chapter 1

## Introduction

Enzymes α-glucosidases in the intestinal lumen and in the brush border membrane play main roles in carbohydrate digestion to degrade starch and oligosaccharides to monosaccharides before they can be absorbed. It was proposed that suppression of the activity of such digestive enzymes would delay the degradation of starch and oligosaccharides, which would in turn cause a decrease in the absorption of glucose and consequently the reduction of postprandial blood glucose level elevation (Puls et al., 1977). Recently, the number of diabetes patients particularly non-insulin dependent diabetes mellitus (NIDDM) increase since food consumption behavior has been changed in favor to fast-foods, which contain refined carbohydrates. As a consequence of the high digestion rate in the upper part of the small intestine a postprandial rise in blood glucose is generally rapid and high following a carbohydrate load. Therefore, to achieve the beneficial therapy of noninsulin-dependent diabetes mellitus the extensive searches for α-glucosidase inhibitors are being carried out worldwide (Chanida et al., 2000; Watanabe et al, 1997; Nishioka et al., 1997; Matsui et al., 1996; ) and a growing number of them are being investigated not only in test animals but also in human clinical trials (Breitmeier et al., 1997; Hirsh et al., 1997; Jenkins et al., 1981). One of the most common sources for these surveys are foodstuffs and thus, this research attempted to isolate inhibitors from various Thai herbs against two carbohydrate digestive enzymes, sucrase prepared from rat intestinal acetone powder and porcine pancreatic α-amylase (PPA). This study also included the isolation and purification of such enzymes inhibitors from mulberry (*Morus alba* Linn.).

Mulberry leaves have long been used to feed silkworms and known as traditional Chinese and Thai medicines to cure and prevent diabetes (Asano et al., 2001; Boonyaprapassorn and Chokchaicharuenporn, 1991). The active compounds including 1-deoxynojirinmycin, a potent inhibitor of mammalian α-glucosidases, found in leaves and stem barks of mulberry trees have long been studied on antidiabetic effect (Asano et al., 1994; Yamada et al., 1993; Hikino et al., 1985; Sharaf et al., 1963, 1964). In the recent years, mulberry tea (Mon tea) made from leaves has been of

great interest in Thailand for new sources of herb teas and is claimed to be an antidiabetic drink. However, no report has previously mentioned on the α-glucosidase inhibitors released from mulberry leaves tea. Therefore, this research also studied the effect of brewing time on dry weight content and maltase and sucrase inhibitory activities in mulberry tea infusion.

In addition, the possible effect of the inhibitors on the human organism using caco-2 model system was next examined. Caco-2 (Chantret et al., 1988; Pinto et al., 1983) has been used as a culture model of human intestinal cells for the studies of drug transports (Boulenc et al., 1993; Cogburn et al., 1991; Hilger et al., 1990) and effect of α-glucosidase inhibitors (Chanida et al., 2001; Toda et al., 2000; Nishioka et al., 1998) because of their ability to express most of the morphological and functional characteristics normally associated with the human intestinal epithelium (Hidalgo et al., 1989). The efficiency of the inhibition of the carbohydrate digestion from mulberry tea infusion can thus be calculated by measurement of the liberated extracellular glucose concentration on the apical side and basal sides of Caco-2 monolayers.

# Chapter 2

## Screening of α-Glucosidase Inhibitors from various Thai herbs

## Introduction

Based on the idea that inhibition of α-glucosidases would delay the degradation of starch and oligosaccharides, which would, in turn, cause a decrease in the absorption of glucose and consequently inhibit the increase in postprandial blood glucose concentration, various kinds of locally collected Thai herbs were screened for inhibitors. Two α-glucosidases, sucrase prepared from rat intestinal acetone powder and porcine pancreatic α-amylase (PPA), were used in this study.

## 2.1 Materials and Methods

### 2.1.1 Materials

Thai herbs were collected locally from lower part of Northern Thailand and were purchased from a local market and traditional medicinal plants store in Phitsanulok, Thailand. Starch azure (RBB-starch) and porcine pancreatic α-amylase were purchased from Sigma Chemical Co., and Glucose B test kit for glucose oxidase method to determine the amount of liberated glucose (Nishioka et al., 1998) was obtained from Wako Pure Chemical Industries, Ltd. An HPLC packed column, Inertsil Silica. 6x250 mm, is a product of GL-Sciences Inc. Other chemicals were of guaranteed or reagent grade from commercial sources.

### 2.1.2 Preparation of rat intestinal sucrase solution

Rat intestinal acetone powder was homogenized in teflon homogenizer in 0.1 M potassium phosphate buffer (pH 7.0) containing 5 mM EDTA and centrifuged at 21,000 g for 60 min. Precipitate obtained from centrifugation was solubilized in the same buffer containing 1.0% Triton-X100 (4$^{o}$C, 60 min) and then centrifuged at 110,000 g for 90 min. Crude enzyme solution obtained from the supernatant was dialyzed against 0.01 M potassium phosphate buffer (pH7.0).

### 2.1.3 Preparation of α-amylase (PPA) solution

Porcine pancreatic α-Amylase (PPA) dissolved in 2.9 M NaCl containing 3 mM $CaCl_2$ was diluted with 0.05 M Tris-HCl buffer (pH 6.9) containing 0.01 M $CaCl_2$ to obtain the final concentration of 2.11 unit/ml and used for PPA inhibitory activity assay.

### 2.1.4 Rat intestinal sucrase inhibitory activity

Sucrose solution (28 mM) prepared in 0.1M potassium phosphate buffer (pH 7) were pre-incubated at 37$^{o}$C for 5 min. The test samples in 50% dimethylsulfoxide (DMSO), and rat intestinal sucrase solution (0.11 unit/mg protein/ml) were added into each assay as shown in Table 2-1. After the enzyme reaction was carried out at 37$^{o}$C for 15 min, Tris-HCl solution (2 M, pH 6.9, 1.5 ml) was added to stop the reaction. The reaction mixture was then passed through a basic alumina column to remove phenolic

or acidic compounds which may interfere the enzymatic glucose quantification and the mixture of 50 μl of the filtrate with 200 μl glucose test kit solution was incubated in Falcon PRO-BIND assay plate at 37°C for 30 min. The absorbance of the reaction solution in each well was measured at 490 nm.

**Table 2-1.** Composition of the reaction mixtures for determining sucrase inhibitory activity.

| | Control (+)[a] | Control (-)[b] | Sample | Blank |
|---|---|---|---|---|
| 28 mM sucrose solution (ml) | 0.4 | 0.8 | 0.4 | 0.8 |
| Test samples (ml)[c] | - | - | 0.2 | 0.2 |
| 50% DMSO (ml) | 0.2 | 0.2 | - | - |
| sucrase solution (ml)[d] | 0.4 | - | 0.4 | - |

[a] defined as 100 % enzyme activity

[b] defined as 0% enzyme activity

[c] test samples in 50% DMSO

[d] 0.011 unit in 1 ml reaction volume

### 2.1.5 Porcine pancreatic α-amylase (PPA) inhibitory activity

Starch azure (2 mg) used as a substrate was suspended in 0.05 M Tris-HCl buffer (pH 6.9) containing 0.01 M $CaCl_2$ and boiled for 5 min. Then, the starch azure solution was pre-incubated at 37°C for 5 min. The test samples in 50% DMSO, and PPA were added into each assay as shown in Table 2-2. The reaction was carried out at 37°C for 10 min and stopped by adding 0.1 ml of 50% acetic acid. The reaction mixture was then centrifuged at 3,000 rpm for 5 min at 4°C. The absorbance of the supernatant at 595 nm was measured. It should be noted that the activity of PPA solution was tested at various concentrations ranging from 0.11 to 0.53 unit/ml and the result shows that the optimum PPA activity was observed at 0.21 unit/ml. Therefore, PPA at 0.21 unit/ml was used in this study.

**Table 2-2.** Composition of the reaction mixtures for determining PPA inhibitory activity.

| | Control (+)[a] | Control (-)[b] | Sample | Blank |
|---|---|---|---|---|
| Starch azure (mg) | 2.0 | 2.0 | 2.0 | 2.0 |
| 0.05 M Tris-HCl buffer (ml) | 0.7 | 0.8 | 0.7 | 0.8 |
| Test samples (ml)[c] | - | - | 0.2 | 0.2 |
| 50% DMSO (ml) | 0.2 | 0.2 | - | - |
| PPA (ml)[d] | 0.1 | - | 0.1 | - |

[a] defined as 100 % enzyme activity

[b] defined as 0% enzyme activity

[c] test samples in 50% DMSO

[d] 0.21 unit in 1ml reaction volume

The $\alpha$-glucosidase inhibitory activity was calculated as follows:

The $\alpha$-glucosidase inhibitory activity (%)

$= [(A_{c+} - A_{c-}) - (A_s - A_b)]/(A_{c+} - A_{c-}) \times 100$

where $A_{c+}$, $A_{c-}$, $A_s$, and $A_b$ are defined as absorbance of 100% enzyme activity (control (+)), 0% enzyme activity (control (-)), test samples, and blank, respectively.

### 2.1.6 Screening for $\alpha$-glucosidase inhibitory activity in Thai herbs.

Forty-seven Thai herbs samples which are *Aloe vera* Linn. (root, leaf), *Schefflera leucantha* Viguier (leaf), *Cassia siamea* Lamk (young leaves), *Tamarindus indica* Linn. (root, leaf, seed, stem bark, pod), *Cocos nucifera* Linn. (flower, aerating root, stem bark, pulp), *Thunbergia laurifolia* Linn. (whole plant), *Citrus hystrix* DC. (root, fruit skin, leaf, fruit), *Coix lachryma-jobi* Linn. Gramineae (root, fruit), *Physalis minima* Linn. Solanaceae (root, stem, leaf) *Amorphophallus campanulatus* Blume (tuber), *Alternanthera sessilis* (Linn.) DC. Amaranthceae (whole plant), *Eclipta prostrata* Linn. (root, stem, leaf), *Ocimum Sanctum* Linn. (leaf, seed), *Oldenlandia biflora* (root, fruit), *Mallotus philippensis* (Lam) Muell Arg (wood, flower, fruit), *Azadirachta indica* A.Jauss (branch, stem bark, flower), *Morus alba* Linn. (leaf, stem bark), *Ocimum basilicum* Linn.

(leaf), *Syzygium cumini* (Linn) Skeels (stem bark), *Strebles asper* Lour (stem bark, wood), *Dendrophthoe pentandra* Miq. (whole plant) and *Saccharum officinarum* Linn (root, stem) were extracted with 50% aqueous MeOH (10 ml/g dry wt.) for 24 h at room temperature. From one part of the extract the solvent was evaporated. The dried residue was redissolved in 50% DMSO and subjected to α-amylase inhibitory activity and sucrase inhibitory activity determination. Thai herb extracts, which showed α-amylase and/or sucrase inhibitory activity more than 50% will be selected for further isolation.

## 2.2 Result and Discussion

Aqueous methanol extracts of forty-seven Thai herbs were screened for α-glucosidase inhibitors and the results are shown in Table 2-3. Thai herbs which showed more than 50% inhibitory activity against both PPA and rat intestinal sucrase enzyme were *Cassia siamea* Lamk; young leaf (100%, 73.87%), *Citrus hystrix* DC.; fruit (100%, 98.57%), *Tamarindus indica* Linn.; pod (98.43%, 96.55%); leaf (96.58%, 85.78%) , *Mallotus philippensis* (Lam) Muell Arg.; flower (100.00%, 52.95%), *Strebles asper* Lour; stem bark (73.50%, 91.56%), *Oldenlandia biflora*; fruit (95.05%, 92.88%), *Morus alba* Linn.; leaf (85.70%, 94.71%), *Syzygium cumini* Linn.; stem bark (94.55%, 98.17%). Among these, *Morus alba* Linn. (Mulberry) leaves showed high α-amylase and sucrase inhibitory activities in ethyl acetate phase after the solvent partition (68.47% and 68.15% respectively) and pH obtained from mulberry extract was 7.3, which pH surely not affected the activity of PPA and sucrase enzymes. In addition, the interest of mulberry leaves tea (Mon tea) as healthy drink is increasing nowadays. Therefore, further work focused on the isolation of the inhibitors from mulberry leaves.

**Table 2-3**. PPA and sucrase inhibitory activities of 50% MeOH extract of various Thai herbs.

| Thai herbs | | Enzymes inhibitory activity (%) | |
|---|---|---|---|
| | | PPA[a] | Sucrase[b] |
| *Eclipta prostrata* Linn. | leaf | 24.01 | 32.93 |
| | root | 9.41 | 0.02 |
| | stem | 7.63 | 1.63 |
| *Ocimum Sanctum* Linn. | seed | - | 20.94 |
| | leaf | 17.98 | 9.50 |
| *Cassia siamea* Lamk. | young leaf | 100.00 | 73.87 |
| *Mallotus philippensis* (Lam) Muell Argb | wood | 59.48 | 34.89 |
| | fruit | - | - |
| | flower | 100.00 | 52.95 |
| *Physalis minima* Linn. | root | 47.43 | - |
| | leaf | 65.89 | 44.09 |
| | stem | - | - |
| *Amorphophallus campanulatus* Blume. | tube | 30.66 | - |
| *Alternanthera sessilis* (Linn.) DC. | whole plant | - | 11.21 |
| *Citrus hystrix* DC. | leaf | 21.39 | 15.91 |
| | root | - | 7.68 |
| | fruit | 100.00 | 98.57 |
| | skin | 1.02 | 33.01 |
| *Cocos nucifera* Linn. | flower | 95.04 | 15.02 |
| | aerating root | 57.04 | 18.87 |
| | stem bark | 78.36 | 0.88 |
| | pulp | 27.32 | - |

**Table 2-3**. PPA and sucrase inhibitory activities of 50% MeOH extract of various Thai herbs (cont.).

| Thai herbs | | Enzymes inhibitory activity (%) | |
|---|---|---|---|
| | | PPA[a] | Sucrase[b] |
| *Tamarindus indica* Linn. | root | 26.67 | - |
| | pod | 98.43 | 96.55 |
| | stem bark | 100.00 | 7.04 |
| | seed | 100.00 | 38.44 |
| | leaf | 96.58 | 85.78 |
| *Thunbergia laurifolia* Linn. | whole plant | 45.15 | 13.66 |
| *Aloe vera* Linn. | root | 45.26 | 47.50 |
| | leaf | 6.66 | 95.50 |
| *Azadirachta indica* A.Jauss | branch | 93.23 | 5.71 |
| | flower | 96.95 | 25.19 |
| | stem bark | 100.00 | 17.09 |
| *Oldenlandia biflora* | root | - | 8.81 |
| | fruit | 95.05 | 92.88 |
| *Morus alba* Linn. | leaf | 85.70 | 94.71 |
| | stem bark | 11.00 | 93.93 |
| *Syzygium cumini* (Linn.) Skeels | stem bark | 94.55 | 98.17 |
| *Ocimum basilicum* Linn. | leaf | - | 33.85 |
| *Schefflera leucantha* Viguier | leaf | 12.59 | 21.63 |
| *Saccharum officinarum* Linn. | root | 81.18 | 4.33 |
| | stem | 21.72 | 100.00 |
| *Coix lachryma-jobi* Linn. Gramineae | root | 66.30 | 23.28 |
| | fruit | 88.97 | 25.73 |
| *Strebles asper* Lour | wood | 78.02 | 34.11 |
| | stem bark | 73.50 | 91.56 |
| *Dendrophthoe pentandra* Miq. | whole plant | 93.53 | 40.58 |

[a] PPA inhibitory activity was determined at concentration of 50 mg/ml reaction mixture.

[b] Sucrase inhibitory activity was determined at concentration of 25 mg/ml reaction mixture.

# Chapter 3

# Isolation and Identification of α-glucosidases Inhibitors from mulberry leaves (*Morus alba* Linn.)

## Introduction

In the previous chapter we found that the ethyl acetate fraction of mulberry leaves (*Morus alba* Linn.) showed high inhibitory activity against both PPA and surase enzymes. In addition, pH obtained from mulberry extract was in active range of α-glucosidases enzyme. Moreover, mulberry tea (Mon tea) has become popular as healthy drink, nowadays. For the above mentioned reasons, mulberry sample was chose to study further on isolation and identification of α-glucosidase inhibitors.

## 3.1 Materials and Methods

### 3.1.1 Isolation of PPA inhibitors from mulberry (*Morus alba* Linn.) leaves

The methanol extract of mulberry leaves was partitioned between ethyl acetate and water. The resulting ethyl acetate was evaporated and redissolved in 50% DMSO and monitored for enzymes inhibitory activities. The active ethyl acetate partitioned fraction was basically purified by Thin Layer and chromatographed on column (Silica, 50 g, 2.0x36.0 cm). The Hexane: Chloroform gradient used was showed in Table 3-1. All fractions were then monitored for PPA and sucrase inhibitory activities. The active eluted fraction from column chromatography was further purified by HPLC (column: Inertsil Silica, 6x250 mm) using mobile phase, hexane-chloroform (10:90) containing 0.1% TFA (v/v); flow rate, 1 ml/min; detection, UV 254 nm. The active fraction was identified based on the enzymes inhibitory activities. The further purification was carried out by HPLC rechromatography using the same condition and the chemical structure of isolated inhibitors was determined by mass-spectoscopy (MS) and nuclear magnetic resonance (NMR) measurements.

### 3.1.2 Instrumental analyses

Field desorption mass spectra (FD-MS) were determined by JEOL JMS-SX-102A instrument. Nuclear magnetic resonance spectra (NMR) were measured by Bruker AMX500 (500 MHz for $^{1}H$ and 125 MHz for $^{13}C$) spectrometer.

### 3.1.3 α-Glucosidase inhibitory assay

The α-Glucosidase inhibitory assay was carried out by the same procedure described in 2.1

**Table 3-1**. Silica column chromatography of 50% MeOH extract of mulberry leaves

Mulberry leaves 50 g; silica gel 50g; column 2.0x36.0 cm

| Fractions | Solvent<br>Hexane : Chloroform | volume |
|---|---|---|
| 1-2 | 40:60 | 50ml x 2 |
| 3-4 | 20:80 | 50ml x 2 |
| 5-6 | 10:90 | 50ml x 2 |
| 7-8 | 0:100 | 50ml x 2 |

## 3.2 Result and Discussion

### 3.2.1 Isolation of PPA inhibitors from mulberry leaves

The 50% aqueous methanol extract of mulberry leaves showed both PPA and sucrase inhibitory activity in ethyl acetate fraction (68.47, and 68.15%, respectively) whereas high sucrase inhibitory activity was only found in water soluble part of mulberry leaves (Fig. 3-1). Therefore, the isolation of PPA inhibitors from the ethyl acetate fraction of mulberry leaves extract was firstly carried out by subjecting to Silica column chromatography followed by preparative HPLC. During the isolation process, collected fractions were further purified on the basis of having PPA inhibitory activity. The eluted fractions No. 5 and 6 obtained from coulumn chromatography with hexane-chloroform gradient presented in Table 3-1 showed highest PPA inhibitory activity (62%). Therefore, the eluted active fractions was further purified by HPLC (column: Inertsil Silica, 6x250 mm) and the HPLC profile is shown in Fig. 3-2. The result revealed that the first active collected fraction which showed the shortest retention time (3.7 min) could be obtained from HPLC using mobile phase, hexane-chloroform (10:90) containing 0.1% TFA (v/v); flow rate, 1 ml/min; detection, UV 254 nm. Its PPA and rat intestinal sucrase inhibitory activity was 71% and 10%, respectively. This indicated that the ethyl acetate fraction of 50% MeOH extract of mulberry leaves expressed inhibitory effect against mainly PPA enzymes.

### 3.2.2 Chemical structure analysis

The active principles obtained by the final HPLC purification showed a typical fatty acid pattern in the $^1$H NMR spectrum which included triplets of terminal methyls at $\delta$ 0.85 and 0.95, a massive undissolved peak of long-chain methylenes at $\delta$1.2-1.4, and characteristic low-field methylenes adjacent to carbonyl around $\delta$ 2.3. The field-desorption mass spectrum of the isolate gave molecular peaks at m/z 256 and 282 (chart 3-1, 3-2). These spectral data indicate the active constituents from mulberry leaves to be a mixture of palmitic and oleic acids.

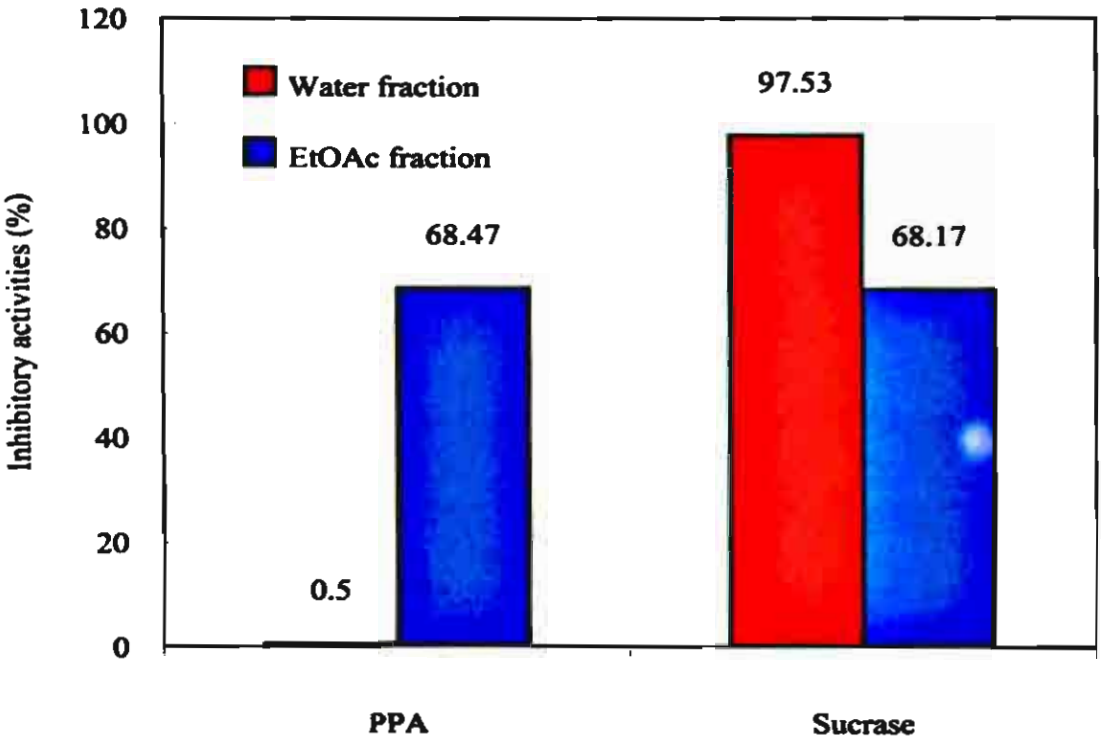
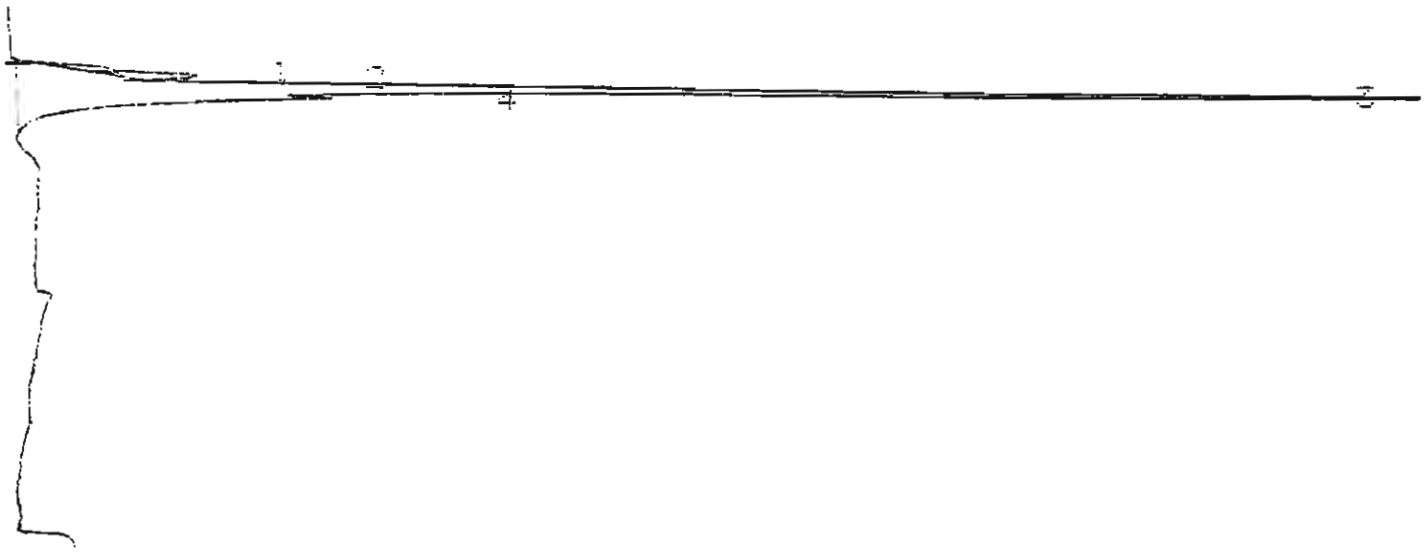



**Fig 3-1. α-Glucosidases inhibitory activities of 50% MeOH extract of mulberry leaves.**



**Column:** Inertsil Silica, 6x250 mm
**Solvent:** hexane-chloroform (10:90)
containing 0.1% TFA (v/v)
**Flow rate:** 1 ml/min
**Detector:** UV 254 nm

**Fig. 3-2.** Preparation and HPLC analysis of ethyl acetate fraction of 50% MeOH extract of mulberry leaves.

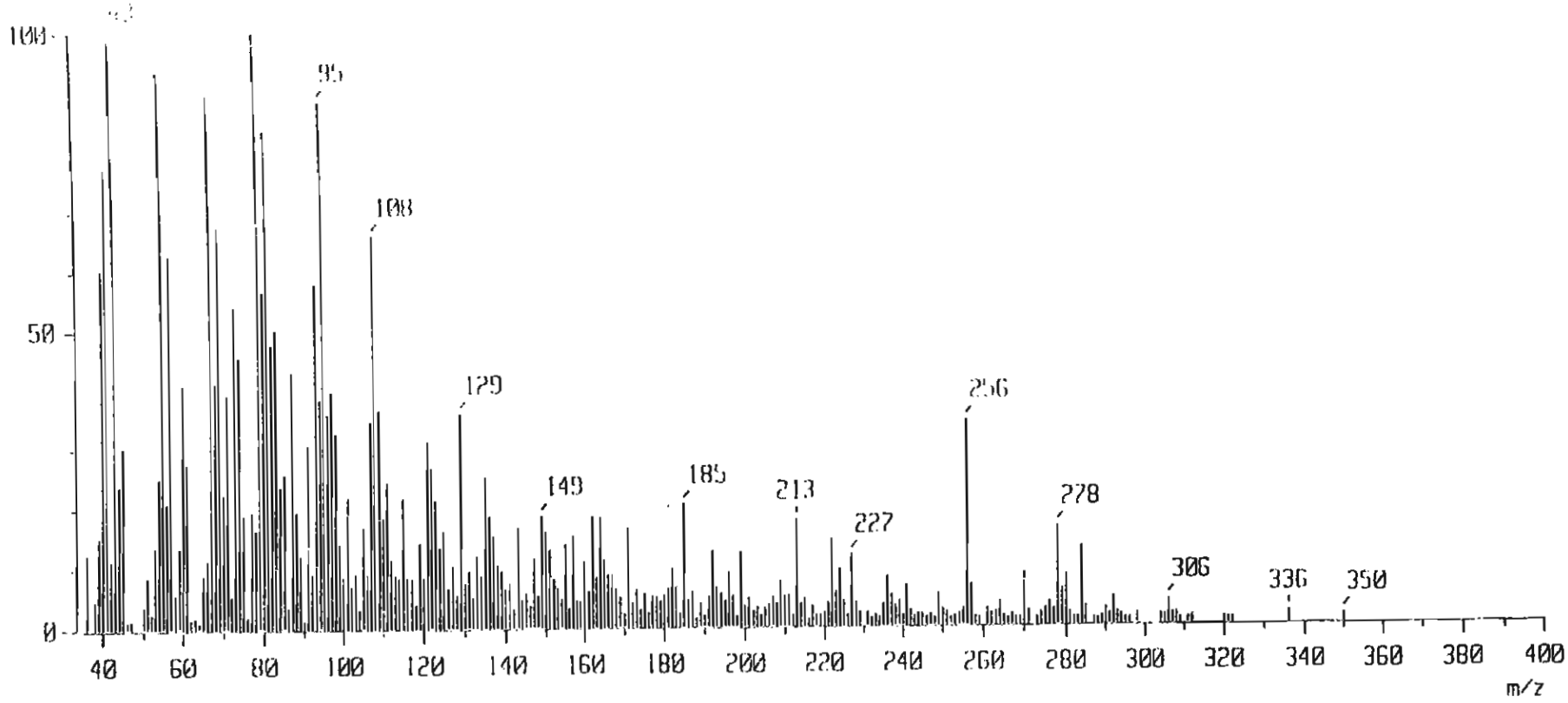



**Chart 3-1.** The FD mass spectrum of the mixture of palmitic and oleic acid isolated from ethyl acetate fraction of 50% MeOH extract of mulberry leaves.

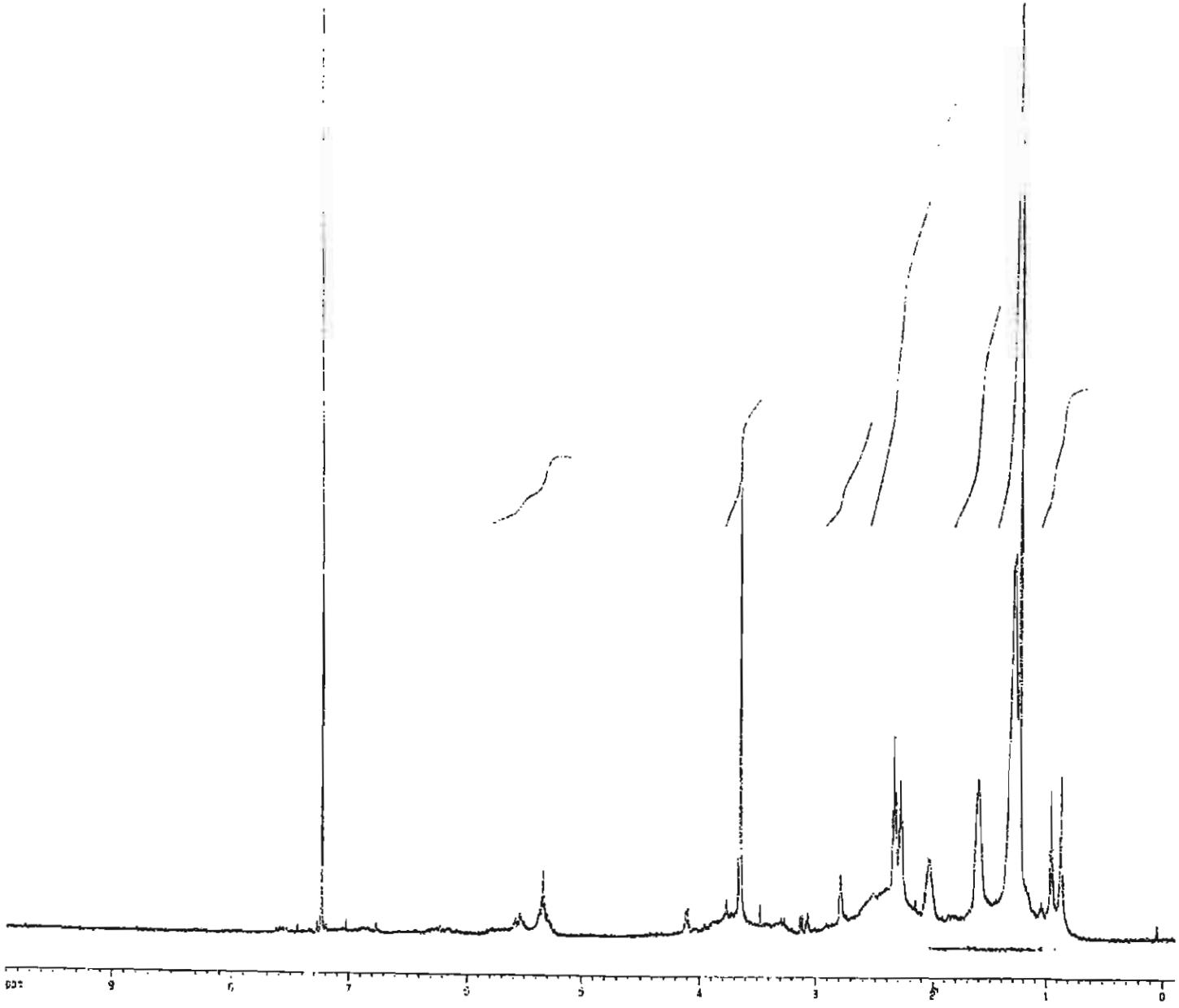


**Chart 3-2**. The $^{1}$H-NMR spectrum of the mixture of palmitic and oleic acid isolated from ethyl acetate fraction of 50% MeOH extract of mulberry leaves.

# Chapter 4

## α-glucosidases inhibitory activities of mulberry tea (Mon tea)

## Introduction

The mixture of palmitic and olecid acid isolated from ethyl acetate fraction of 50% MeOH extract of mulberry leaves showed 71% PPA inhibitory activity. However, the result showed that high sucrase inhibitory activity (97.53%) was also found in water fraction of mulberry leaves extract. Moreover, in the recent year's mulberry leaves has been consumed as herb tea in Thailand, which is claimed to have antidiabetic effect. The potent inhibitors against α-glucosidases enzyme including 1-deoxynojirimycin has long been isolated from aqueous fraction of mulberry leaves, stem bark, and root and were studied on their hypoglycemic activity. Therefore, in this study, the isolation of such inhibitors from water fraction of mulberry leaves was omitted. Instead, the α-glucosidases, sucrase and maltase enzymes, inhibitory activities of hot water extract of mulberry leaves in variation of products and brewing time was studied.

## 4.1 Materials and Methods

### 4.1.1 Materials.

Mulberry leaves, product A, were collected locally in Phitsanulok and dry in hot air oven at 45°C for 24 h. Product B, C, and D are products of mulberry leaf tea (Mon tea) which were purchased from local market in Northern part of Thailand.

### 4.1.2 Hot water extraction of mulberry leaves and tea products

The method of hot water extraction was modified (Phillips, 1996). Briefly, hot water (95°C) of 100 ml was added to 1 g of mulberry leaves and mulberry tea products and the mixture was allowed to stand for brewing time of 3, 5, 7, 10, and 30 min. The tea infusion was then filtered and subjected to dry weight content and α-glucosidases inhibitory activity determinations.

### 4.1.3 Preparation of rat intestinal sucrase and maltase solution

Rat intestinal acetone powder was homogenized in teflon homogenizer in 0.1 M potassium phosphate buffer (pH 7.0) containing 5 mM EDTA and centrifuged at 21,000 g for 60 min. Precipitate obtained from centrifugation was solubilized in the same buffer containing 1.0% Triton-X100 (4°C, 60 min) and then centrifuged at 110,000 g for 90 min Crude enzyme solution obtained from the supernatant was dialyzed against 0.01 M potassium phosphate buffer (pH7.0).

### 4.1.4 Rat intestinal sucrase inhibitory activity

The sucrase inhibitory assay was carried out by the same procedure described in 2.1.4

### 4.1.5 Rat intestinal maltase inhibitory activity

Maltose solution (28 mM) prepared in 0.1M potassium phosphate buffer (pH 7) were pre-incubated at 37°C for 5 min. The test samples in 50% dimethylsulfoxide (DMSO), and rat intestinal maltase solution (0.49 unit/mg protein/ml) were added into each assay as shown in Table 4-1. After the enzyme reaction was carried out at 37°C for 15 min, Tris-HCl solution (2 M, pH 6.9, 1.5 ml) was added to stop the reaction. The

reaction mixture was then passed through a basic alumina column to remove phenolic or acidic compounds which may interfere the enzymatic glucose quantification and the mixture of 50 μl of the filtrate with 200 μl glucose test kit solution was incubated in Falcon PRO-BIND assay plate at 37°C for 30 min. The absorbance of the reaction solution in each well was measured at 490 nm.

**Table 4-1.** Composition of the reaction mixtures for determining maltase inhibitory activity.

| | Control (+)[a] | Control (-)[b] | Sample | Blank |
|---|---|---|---|---|
| 28 mM sucrose solution (ml) | 0.7 | 0.8 | 0.7 | 0.8 |
| Test samples (ml)[c] | - | - | 0.2 | 0.2 |
| 50% DMSO (ml) | 0.2 | 0.2 | - | - |
| maltase solution (ml)[d] | 0.1 | - | 0.1 | - |

[a] defined as 100 % enzyme activity

[b] defined as 0% enzyme activity

[c] test samples in 50% DMSO

[d] 0.049 unit in 1 ml reaction volume

The α-glucosidase inhibitory activity was calculated as follows:

The α-glucosidase inhibitory activity (%)
$= [(A_{c+}-A_{c-}) - (A_s - A_b)]/(A_{c+} - A_{c-}) \times 100$

where $A_{c+}$, $A_{c-}$, $A_s$, and $A_b$ are defined as absorbance of 100% enzyme activity (control (+)), 0% enzyme activity (control (-)), test samples, and blank, respectively.

### 4.1.6 Statistical Analysis

Variance analysis of the data was performed by using the software SPSS for windows version 10.

## 4.2 Result and Discussion

### 4.2.1 Sucrase and maltase inhibitory activities of water fraction of 50% MeOH extract of mulberry leaves.

In order to study α-glucosidase inhibitory effect of hot water extract of mulberry leaves, the $IC_{50}$ values, the concentration of 50% enzyme inhibition obtained, of the aqueous fraction of mulberry leaves extract was firstly determined. Mulberry leaves was extracted with 50% MeOH (10 ml/g dry wt.) for 24 h at room temperature 2 or 3 times. The supernatant of the extract was obtained by filtration with cotton. The extract was then partitioned between ethyl acetate and water. The resulting water solutions was evaporated and then, redissolved in 50% DMSO (10 ml/g dry wt.) and submitted to sucrase and maltase inhibitory activities and result showed in Fig. 4-1. The $IC_{50}$ values of sucrase and maltase inhibition were 0.003 mg/ml and 0.008 mg/ml, respectively. It can be seen that their $IC_{50}$ values are quite low and that of sucrase inhibition was almost 3 times lower than maltase inhibition. This showed the similar result with the previous report which found that acarbose, glucosidase inhibitor as commecial medicine, express $IC_{50}$ values of sucrase inhibition lower than that of maltase inhibition (Matsui et al., 2001). Therefore, the concentration used for comparative study of sucrase and maltase inhibitory activity in hot water extract of mulberry leaves and mulberry tea products were 0.005 mg/ml and 0.02 mg/ml, respectively. These might be in the dose range for inhibition occurred.

### 4.2.2 The effect of brewing time on sucrase and maltase inhibitory activities in mulberry tea products

To study the potential possibility to obtain the healthy drink from mulberry leaves, hot water extraction of mulberry leaves and mulberry tea products were performed by modification of tea preparation. Tea infusion (1g of leaf tea/100 ml hot water, 95°C) prepared from locally collected mulberry leaves (product A) and commercial products of mulberry leaf tea (product B, C, and D) were determined for dry weight content (mg/ml) and α-glucosidase inhibitory activity at brewing time of 3, 5, 7, 10, and 30 min. Sucrase and maltase inhibitory activities were measured at concentration of 0.005 mg/ml and 0.02 mg/ml, respectively.

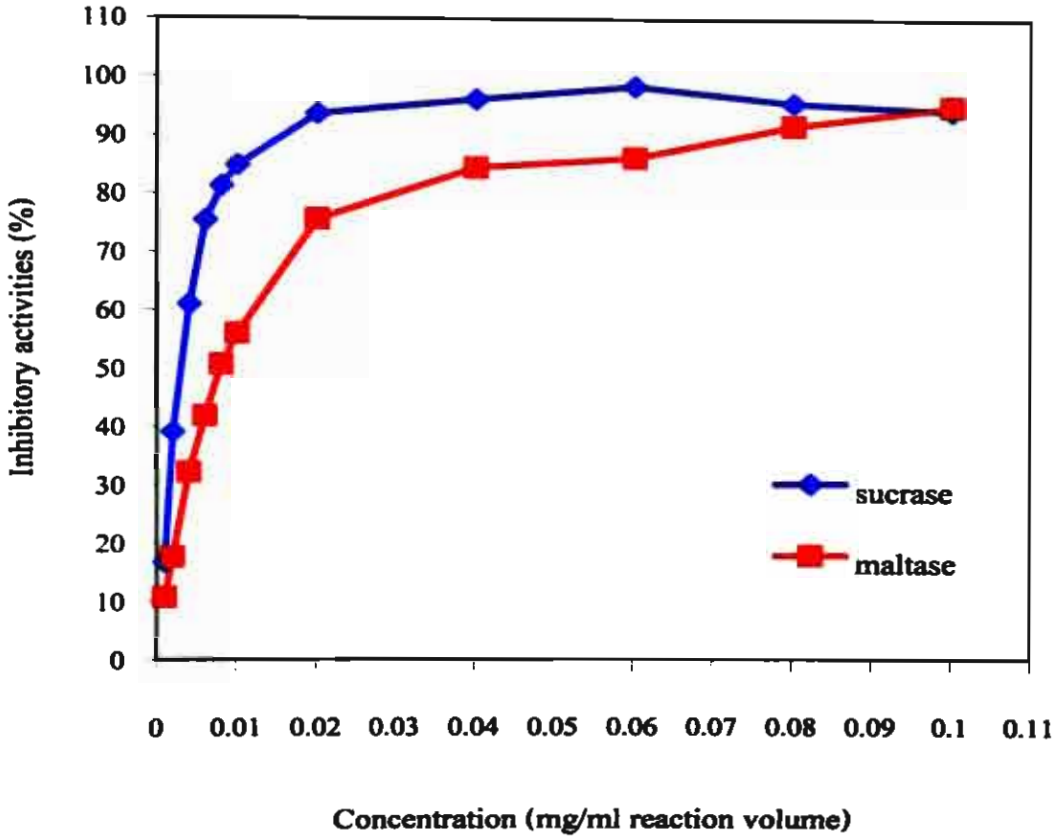



**Fig. 4-1.** Sucrase and maltase inhibitory activities of water fraction of 50% MeOH of mulberry leaves.

The dry weight content was slightly higher in longer brewing time and most of the products showed the highest dry weight content at 30 min (Table 4-2). This indicates that the longer infusion time the more amounts of compounds can be extracted from mulberry tea. However, the highest sucrase inhibitory activity could be obtained from different brewing time in each product while the highest maltase inhibitory activity could mostly be found in 5 min infusion (Table 4-3, 4-4). It can be conclude that the content of active compound extracted by hot water was not in proportional to brewing time. In order to study the difference of α-glucosidase inhibitory activity in various types of mulberry tea products, sucrase and maltase inhibitory activities of tea products were compared at each brewing time and the result showed the significance difference among products (Table 4-5, 4-6). It can be seen that product C showed the highest inhibitory activity against both sucrase and maltase at 3 min infusion, while product A gave the highest inhibitory activity at 5 and 7 min. At infusion time of 10 and 30 min, the highest sucrase inhibitory activity was obtained from B and the highest maltase inhibitory activity was found in product B and C, respectively. Product D showed the most less potent inhibitory activity against both sucrase and maltase enzymes. Since product A is collected mulberry leaves and it was dried in hot air oven at 45$^{\circ}$C for 24 h and then, directly subjected to hot water extraction whereas product B, C, and D are commercial teas probably prepared by different methods. It implied that the preparation of leaf tea and growing area of mulberry trees might be contributed to the α-glucosidase inhibitory effects. However, it can conclude that infusion time of 3-5 min for tea preparation can be sufficient to obtain healthy drink for antidiabetic purpose.

**Table 4-2.** Dry weight content of mulberry tea infusions at various brewing times

| Brewing time (min) | Dry weight content (mg/ml) | | | |
|---|---|---|---|---|
| | A | B | C | D |
| 3 | 1.08 | 1.08 | 1.50 | 1.38 |
| 5 | 1.61 | 1.50 | 1.74 | 1.42 |
| 7 | 1.72 | 1.62 | 1.82 | 1.70 |
| 10 | 1.96 | 2.08 | 1.92 | 1.54 |
| 30 | 2.74 | 2.72 | 2.24 | 1.38 |

Tea infusions were prepared from 1 g mulberry tea in 100 ml hot water (98$^{o}$C).

**Table 4-3.** Sucrase inhibitory activity of tea infusions at various brewing time

| Brewing time (min) | Sucrase inhibitory activity (%) | | | |
|---|---|---|---|---|
| | A | B | C | D |
| 3 | 44.27b | 40.77a | 47.37a | 32.80b |
| 5 | 73.70d | 57.93d | 44.76a | 33.91b |
| 7 | 62.58c | 58.01d | 47.61a | 29.01a |
| 10 | 37.03a | 53.40c | 48.18a | 31.77ab |
| 30 | 43.31b | 49.95b | 43.82a | 34.94b |

Note: Sucrase inhibitory activity was determined at concentration of 0.005 mg/ml. Tea infusions (1g tea/100 ml hot water) were prepared from dried mulberry leaves: A, mulberry teas from different brand name products B, C, and D. Values within a column with the different letters are significantly different ($P<0.05$)

**Table 4-4**. Maltase inhibitory activity of mulberry tea infusions at various brewing time

| Brewing time (min) | Maltase inhibitory activity (%) | | | |
|---|---|---|---|---|
| | A | B | C | D |
| 3 | 47.20b | 46.40a | 47.10a | 39.75e |
| 5 | 67.10d | 57.45e | 47.55b | 39.15d |
| 7 | 60.40c | 56.80d | 48.80c | 35.25a |
| 10 | 42.10a | 55.05c | 50.15d | 37.85c |
| 30 | 47.25b | 51.95b | 53.85e | 37.55b |

Note: Maltase inhibitory activity was determined at concentration of 0.02 mg/ml. Tea infusions (1g tea/100 ml hot water) were prepared from dried mulberry leaves: A, mulberry teas from different brand name products B, C, and D. Values within a column with the different letters are significantly different ($P<0.05$)

**Table 4-5**. Sucrase inhibitory activity of different mulberry tea products at each brewing time

| Tea products | Sucrase inhibitory activity | | | | |
|---|---|---|---|---|---|
| | Brewing time (min) | | | | |
| | 3 | 5 | 7 | 10 | 30 |
| A | 44.27c | 73.70d | 62.58d | 37.03b | 43.31b |
| B | 40.77b | 57.93c | 58.01c | 53.40d | 49.95c |
| C | 47.37d | 44.76b | 47.61b | 48.18c | 43.82b |
| D | 32.80a | 33.91a | 29.01a | 31.77a | 34.94a |

Note: Sucrase inhibitory activity was determined at concentration of 0.005 mg/ml. Tea infusions (1g tea/100 ml hot water) were prepared from dried mulberry leaves: A, mulberry teas from different brand name products B, C, and D. Values within a column with the different letters are significantly different ($P<0.05$).

**Table 4-6**. Maltase inhibitory activity of different mulberry tea products at each brewing time

| Tea products | Maltase inhibitory activity | | | | |
|---|---|---|---|---|---|
| | Brewing time (min) | | | | |
| | 3 | 5 | 7 | 10 | 30 |
| A | 47.20c | 68.05d | 60.40d | 42.10b | 47.25b |
| B | 46.40b | 57.45c | 56.80c | 55.05d | 51.95c |
| C | 47.10c | 47.55b | 48.80b | 50.15c | 53.85d |
| D | 39.75a | 39.15a | 35.25a | 37.85a | 37.55a |

Note: Maltase inhibitory activity was determined at concentration of 0.02 mg/ml. Tea infusions (1g tea/100 ml hot water) were prepared from dried mulberry leaves: A, mulberry teas from different brand name products B, C, and D. Values within a column with the different letters are significantly different ($P<0.05$).

# Chapter 5

## Studies on α-glucosidases inhibitory activity of hot water extract of mulberry tea using Caco-2 model system

## Introduction

In the previous chapter we found that hot water extract of mulberry leaves showed the potent inhibitory effect against carbohydrate digestive enzymes, sucrase and maltase. Moreover, it was known that in the preparation of mulberry tea, brewing time must be considered in order to get the most effective for antidiabetic purpose. However, the possible inhibition effects of mulberry tea on the human organism can not be judged completely. Therefore, a model system to study the inhibition of intestinal glucosidase enzymes using Caco-2 monolayer was performed. The cell experiment design was basically adopted from the studies of drug transports using the human Caco-2 monolayers (Boulenc et al., 1993; Cogburn et al., 1991; Hidalgo et al., 1989).

## 5.1 Materials and Methods

### 5.1.1 Preparation of culture medium

Powder of Dulbecco's modified Eagle's minimum essential medium (DMEM, Gibco BRL, 13.5 g), streptomycin sulfate (100 mg), gentamycin sulfate (50 mg), pennicilin G potassium (100,000 unit) were dissolved in MilliQ water (940 ml). $NaHCO_3$ solution (7.5% (w/v), 50 ml), MEM non-essential amino acids solution (10 ml), and 20% (v/v) fetal bovine serum (FBS) (Biosciences PTL LTD.) were added in the medium solution.

### 5.1.2 Cell culture

Caco-2 cells were obtained from the American Type Culture Collection (passage 31). Cells were routinely cultured in T-75 tissue culture flasks (Falcon) at 37 $^{o}$C in an atmosphere containing 5% $CO_2$. At 70-80% confluence (4-5 days after culture in flask), cells were removed from the flasks by incubation with 2 ml of 0.025% trypsin solution containing 0.02% EDTA for 2-5 min. Cells were resuspended in fresh medium to inhibit trypsin activity, collected by centrifugation (100xg, 5 min), resuspended in fresh medium, and then they were equally distributed into new culture vessel. The culture medium was changed every 2 days regardless of the culture condition during the investigation.

### 5.1.3 Caco-2 enzyme activity measurements

#### 5.1.3.1 TEER measurement.

The health and confluency of Caco-2 cells were evaluated by measuring transepithelial electrical resistance (TEER) (Artursson, 1990). The device utilized in obtaining TEER values has been described previously (Furie et al., 1987). Current pulses of 100 μA were passed through two electrodes made of 1.2 mm glass capillary tubing containing 3.0 M KCl and 3% agar, connected to Ag/AgCl pellet electrodes (Millipore, Millicell-ERS) and voltage changes were detected with a second pair of electrodes. Electrical resistance values were calculated by multiplying the voltage changes by the surface area of the polyethylene terephthalate (PET, Falcon) membrane (4.2 $cm^2$), on which the cells were grown, and then applying Ohm's law. Transepithelial electrical resistance values obtained in the absence of cells (caused by the electrical system and the PET membrane) were considered as blank. Before measuring of TEER

value the electrodes were cleaned with 70% EtOH and were left standing in the culture medium (DMEM) for 15 min. Then, electrodes were immersed into the wells of cell culture plate with cell monolayer (sample) and without cell monolayers (blank) and the resistance was recorded.

TEER value was calculated by the equation below.

$$\text{TEER value } (\Omega x cm^2) = (Rs\text{-}Rb) \times \text{growth area of membrane}$$

Where Rs = resistance of sample

Rb = resistance of blank

(Rs-Rb) = resistance of cell monolayer

### 5.1.3.2 Sucrase-maltase activity

Sucrase-maltase activity of cells grown on cell culture inserts of 6 well cell culture plates were detemined every 3 days after seeding. Cells were washed with phosphate-buffered saline (PBS) both at the apical and the basal sides 2 or 3 times. Cells were scraped out by using spatula and resuspended in 1.5 ml of fresh PBS. For sucrase activity measurement, 0.4 ml of homogenized cells were added into 0.6 ml of 28 mM sucrose solution while 0.1 ml of homogenized cells were added into 0.9 ml of 28 mM maltose solution in the case of maltase activity measurement. Each reaction mixture was then incubated at 37°C for 50 min. The reaction was stopped by immersing the reaction tubes into boiling water for 5 min. Then, 50 µl of the reaction solution with 200 µl glucose test kit (Glucose B test kit) solution was mixed and incubated in Falcon PRO-BIND assay plate at 37°C for 30 min. Then, the absorbance of the mixture was measured at 490 nm. The total protein content of homogenized cells was determined by the Bradford method (Marion, 1976). Sucrase and maltase specific activities were calculated as enzyme unit/mg total protein.

### 5.1.4 Mulberry tea preparation.

Mulberry tea infusion was prepared by adding deionized water (95°C) of 100 ml to 1 g of mulberry leaves and mulberry tea products and the mixture was allowed to stand for appropriate brewing time of each product. The brewing time of mulberry tea infusion used in this study was showed in Table. 5-1. The tea infusion was then filtered

and subjected to the apical side of Caco-2 monolayer for α-glucosidases inhibitory activity determinations.

**Table 5-1.** The brewing time of mulberry tea infusion used for sucrase and maltase inhibition determination in Caco-2 cell studied system.

| Mulberry tea products | Brewing time for inhibitory activity determinations (min) | |
|---|---|---|
| | Sucrase | maltase |
| A | 5 | 5 |
| B | 5 | 5 |
| C | 3 | 30 |
| D | 3 | 30 |

### 5.1.5 Sample analysis

In order to study the inhibition of carbohydrate digestion of mulberry tea using Caco-2 monolayers, cells were fed into PET membrane (pore size: 0.4 microns; pore density $1.6x10^6$ pores/$cm^2$; diameter 23.1 mm; in a 6 well plate. After cells reached 100% confluence (5 days), cells were grown more for 14 to 20 days. Then, cell culture medium was removed and both the apical and basal chambers were washed 3 times with 2 ml of PBS. The culture medium in the apical chamber was substituted with a reaction mixture consisted of 28mM sucrose solution in PBS (0.8 ml) and 28mM maltose solution in PBS (0.8 ml) as substrates, and mulberry tea infusion (0.2 ml). In the basal chamber 1 ml of PBS was added instead of the culture medium (Fig. 5-1). The composition of reaction mixture in the cell culture inserts for sucrase and maltase inhibitory activity determination is shown in Table 5-2 and Table 5-3. To check the applicability of this model system and compare the inhibitory effect of mulberry tea to commercial α-glucosidase inhibitor, 1-deoxynojirimycin (Asano et al., 1994; Romero et al., 1985) known as a potent medicine for diabetes was used as a positive control. The assay plate was then incubated at 37°C in 5% $CO_2$ atmosphere for 2 h. After incubation the liberated extracellular glucose concentration in the cell free solution of both apical and basal chamber was measured by the glucose oxidase method as in Chapter 2.

**Table 5-2.** Composition of the reaction mixtures for determining sucrase inhibitory activity in the apical chamber of Caco-2 monolayers

| | Control [a] | Sample [b] | Blank |
|---|---|---|---|
| 28 mM sucrose solution | | | |
| in PBS (ml) | 0.8 | 0.8 | 1.0 |
| Test samples (ml) | - | 0.2 | - |
| PBS (ml) | 0.2 | - | - |

[a] defined as 100 % enzyme activity

[b] test samples in deionized water

**Table 5-3.** Composition of the reaction mixtures for determining maltase inhibitory activity in the apical chamber of Caco-2 monolayers

| | Control [a] | Sample [b] | Blank |
|---|---|---|---|
| 28 mM maltose solution | | | |
| in PBS (ml) | 0.8 | 0.8 | 1.0 |
| Test samples (ml) | - | 0.2 | - |
| PBS (ml) | 0.2 | - | - |

[a] defined as 100 % enzyme activity

[b] test samples in deionized water

The α-glucosidase inhibitory activity was calculated as follows:

The α-glucosidase inhibitory activity (%)

$= [(A_a - A_b) - (A_s - A_b)]/(A_a - A_b) \times 100$

where $A_a$, $A_s$, and $A_b$ are defined as absorbance of 100% enzyme activity (control[a]), test samples, and blank, respectively.