การดูดซึมแกมมา-โอรีซานอลโดยใช้เซลล์เพาะเลี้ยงเยื่อบุลำไส้ชนิดเคโกะ-ทูและฤทธิ์ต้านการเกิดอนุมูลอิสระในไลโปโปรตีนชนิดความหนาแน่นต่ำ

นางกิตณา แมคีเน็น

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีวเวชศาสตร์ (สหสาขาวิชา)
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ปีการศึกษา 2552

BIOAVAILABILITY OF γ-ORYZANOL USING CACO-2 CELLS CULTURE MODEL AND ITS ANTIOXIDANT ACTIVITIES AGAINST LOW- DENSITY LIPOPROTEIN OXIDATION

Mrs. Kittana Mäkynen

A Dissertation Submitted in Partial Fulfillment of the Requirements

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Thesis Title	BIOAVAILABILITY OF γ-ORYZANOL USING CACO-2 CELLS CULTURE MODEL AND ITS ANTIOXIDANT ACTIVITIES AGAINST LOW- DENSITY LIPOPROTEIN OXIDATION
By	Mrs. Kittana Mäkynen
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Thesis Advisor	Assistant Professor Tipayanate Ariyapitipun, Ph.D.

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กิตณา แมคีเน็น : การดูดซึมแกมมา-โอรีซานอลโดยใช้เซลล์เพาะเลี้ยงเยื่อบุลำไส้ชนิดเคโกะ-ทู และฤทธิ์ต้านการเกิดอนุมูลอิสระในไลโปโปรตีนชนิดความหนาแน่นต่ำ. (BIOAVAILABILITY OF γ-ORYZANOL USING CACO-2 CELLS CULTURE MODEL AND ITS ANTIOXIDANT ACTIVITIES AGAINST LOW- DENSITY LIPOPROTEIN OXIDATION) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ. ดร.ทิพยเนตร อริยปิติพันธ์, 146 หน้า.

แกมมา-โอรีซานอลเป็นสารไฟโตสเตอรอลชนิดหนึ่งที่พบในรำข้าวและน้ำมันรำข้าวเท่านั้น มีองค์ประกอบเป็นสารไตรเทอร์ปีน 10 ชนิด ที่จับกับกรดเฟอรูลิก มีรายงานการวิจัยในสัตว์ทดลองและในมนุษย์ถึงความสามารถของแกมมา-โอรีซานอลในการลดระดับคอเลสเตอรอลในเลือด รวมถึงฤทธิ์ต้านอนุมูลอิสระในหลอดทดลอง การวิจัยนี้มีจุดประสงค์เพื่อศึกษากระบวนการลดคอเลสเตอรอลของแกมมา-โอรีซานอลและการต้านการเกิดออกซิเดชันจากอนุมูลอิสระในไลโปโปรตีนชนิดความหนาแน่นต่ำ (แอลดีแอล) โดยทำการศึกษาการดูดซึมแกมมา-โอรีซานอลเข้าสู่เซลล์เยื่อบุลำไส้ชนิดเคโกะ-ทู การยับยั้งคอเลสเตอรอลเข้าสู่ไมเซลล์สังเคราะห์ในหลอดทดลองและในไมเซลล์จากระบบย่อยอาหารจำลองตั้งแต่ปาก กระเพาะอาหาร และลำไส้เล็ก ตลอดจนปริมาณคอเลสเตอรอลที่สามารถดูดซึมเข้าสู่เซลล์เยื่อบุลำไส้เมื่อมีแกมมา-โอรีซานอลอยู่ในระบบที่ความเข้มข้นต่าง ๆ พบว่าแกมมา-โอรีซานอลสามารถย่อยและเข้าสู่ไมเซลล์ในระบบย่อยอาหารจำลองได้ดี แกมมา-โอรีซานอลในระดับความเข้มข้น 1500 ไมโครโมลาร์ ไม่สามารถลดการเข้าสู่ไมเซลล์ของคอเลสเตอรอลได้ที่ระดับความเข้มข้นคอเลสเตอรอล 400 ไมโครโมลาร์ ความเข้มข้นของแกมมา-โอรีซานอลที่ระดับต่ำๆ (13 และ 26 ไมโครโมลาร์) แม้มีสัดส่วนโมลาร์ของแกมมา-โอรีซานอลต่อคอเลสเตอรอลสูงถึง 10 และ 20 เท่า ไม่มีผลในการลดความสามารถของคอเลสเตอรอลเข้าสู่ไมเซลล์ในระบบย่อยอาหารจำลอง แม้ว่าแกมมา-โอรีซานอลที่สัดส่วนโมลาร์ 20 เท่าของคอเลสเตอรอลสามารถลดการดูดซึมคอเลสเตอรอลเข้าสู่เซลล์เยื่อบุลำไส้ได้อย่างมีนัยสำคัญทางสถิติ (p<0.05) นอกจากนี้พบว่าแกมมา-โอรีซานอลสามารถยับยั้งการทำงานของเอนไซม์ HMG-CoA reductase ซึ่งเป็นเอนไซม์สำคัญในการสังเคราะห์คอเลสเตอรอลในร่างกายได้เทียบเท่ากับยาซิมวาสเตติน ส่วนผลการทดสอบฤทธิ์ต้านการเกิดออกซิเดชันในแอลดีแอลที่แยกได้จากอาสาสมัครสุขภาพดี จากการวิเคราะห์ค่า conjugated dienes, thiobarbituric acid-reactive substances (TBARS), relative electrophoresis mobility (REM) และ apo B-100 fragmentation หลังจากกระตุ้นแอลดีแอลให้เกิดออกซิเดชันเป็นเวลา 4 ชั่วโมง พบว่าแกมมา-โอรีซานอลสามารถลดการเกิดออกซิเดชันในแอลดีแอลได้ดีในทุกการทดสอบ และมีฤทธิ์แรงกว่ากรดเฟรูลิกและแอลฟา-โทโคฟีรอลในการยับยั้งการเกิด TBARS มีฤทธิ์เสมอกันกับกรดเฟรูลิกและแอลฟา-โทโคฟีรอลในการทดสอบ REM แต่มีฤทธิ์อ่อนกว่ากรดเฟรูลิกและแอลฟา-โทโคฟีรอล ในการยับยั้งการเกิด conjugated dienes และ apo B-100 fragmentation ดังนั้น จากการศึกษานี้พบว่าแกมมา-โอรีซานอลมีความสามารถในการต้านการเกิดออกซิเดชันในแอลดีแอลได้ดี และเป็นไปได้ว่าความสามารถของแกมมา-โอรีซานอลในการลดคอเลสเตอรอลในเลือดนั้น ไม่เกิดจากการที่แกมมา-โอรีซานอลขัดขวางคอเลสเตอรอลเข้าสู่ไมเซลล์ระหว่างกระบวนการย่อยอาหาร แต่อาจเกิดจากการลดการสังเคราะห์คอเลสเตอรอลในร่างกายโดยยับยั้งการทำงานของเอนไซม์ HMG-CoA reductase อย่างไรก็ตาม ควรมีการวิจัยเชิงลึกเพิ่มเติมระดับยีนต่อไปเพื่อให้เข้าใจกลไกโดยรวมทั้งหมด

สาขาวิชา ชีวเวชศาสตร์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2552 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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KEYWORDS : bioavailability / oryzanol / Caco-2 cells / micelles / LDL oxidation

KITTANA MÄKYNEN: BIOAVAILABILITY OF γ-ORYZANOL USING CACO-2 CELLS CULTURE MODEL AND ITS ANTIOXIDANT ACTIVITIES AGAINST LOW - DENSITY LIPOPROTEIN OXIDATION. THESIS ADVISOR: ASST. PROF. TIPAYANATE ARIYAPITIPUN, Ph.D., 146 pp.

Gamma-oryzanol (γ-OR) is a unique phytosterol in rice bran and its oil. It is a mixture of 10 distinct triterpene derived compounds that are esterified to ferulic acid. Hypocholesterolemic of rice bran oil and γ-OR in animal and human studies have been reported. This study aimed to investigate the proposed mechanism of γ-OR on hypocholesterolemic and antioxidant activities of γ-OR against LDL oxidation. The effects of γ-OR on the cholesterol micellarization, cholesterol uptake by Caco-2 intestinal cells, and HMG-CoA reductase activity were determined. The results showed that γ-OR incorporated efficiently into micelles during simulated digestion of rice meal mixed with high γ-OR oil. High concentration of γ-OR at 1500 μM did not decrease the efficiency of micellarization of cholesterol at 400 μM in synthesis micelles. At 10- and 20-fold molar ratios to cholesterol but low concentration (13 and 26 μM), γ-OR did not inhibit the efficiency of cholesterol micellarization during simulated digestion. Nevertheless, γ-OR at 20-fold molar ratio to cholesterol significantly decreased cholesterol uptake by Caco-2 intestinal cells ($p<0.05$). In addition, γ-OR showed the inhibitory effect on the HMG-CoA reductase activity similar to simvastatin. Furthermore, the antioxidant activities of γ-OR against LDL oxidation were investigated in seven healthy volunteers. Isolated LDL fractions were prepared and the measurement of conjugated dienes, thiobarbituric acid-reactive substances (TBARS) formation, relative electrophoresis mobility (REM), and apo B-100 fragmentation were assessed with varies concentrations of γ-OR compared with ferulic acid and α-tocopherol after initiation of *in vitro* oxidation for 4 hours. This *in vitro* study demonstrated that γ-OR was a good antioxidant against LDL oxidation. Gamma-oryzanol provided stronger antioxidative activity than those of ferulic acid and α-tocopherol in decreasing TBARS formation, and the same as that of ferulic acid and α-tocopherol in the REM test ($p<0.05$). Furthermore, γ-OR was good at prolonging the lag time of conjugated diene formation, and reducing apo B-100 fragmentation, although it was weaker than those of ferulic acid and α-tocopherol ($p<0.05$). These findings suggest that γ-OR provide the strong antioxidative activity against LDL oxidation. Moreover, hypocholesterolemic activity of γ-OR is supposedly due to the inhibition of cholesterol biosynthesis, rather than the inhibition of cholesterol micellarization. However, further study is still needed in nutrigenomic approach to understand the whole hypocholesterolemic mechanism.

Field of Study : Biomedical Sciences Student's Signature .....

Academic Year : 2009 Advisor's Signature .....

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LIST OF ABBREVIATIONS

AAPH	2,2'-azobis (2-mehylpropionamide) dihydrochloride
AMVN	2,2'-azobis (2,4-dimethylvaleronitrile)
$ABTS^{\bullet+}$	2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BMI	Body Mass Index
C2BBe1	A brush border expressing cell line (ATCC CRL-2102) that be cloned from the Caco-2 cell line, form a polarized monolayer with an apical brush border morphologically comparable to that of the human colon
Caco-2	An immortalized cell line (ATCC HTB-37) of hetero-geneous human epithelial colorectal adenocarcinoma cells, widely used for intestinal absorption model
Conc.	Concentration
Chol	Cholesterol
$DPPH^{\bullet}$	2,2'-diphynyl-1-picrylhydrazyl radical
FAO	Food and Agriculture Organization
γ-OR	Gamma-oryzanol
GC-MS	Gas chromatography-mass spectrometry
HDL-C	High density lipoprotein-cholesterol
HMG-CoA reductase	3-hydroxy-3-methyl-glutaryl-CoA reductase
IgE	Immunoglobulin isotype E, a class of antibody, plays an important role in allergy
IL-2	Interleukin-2, a type of cytokine immune system signaling molecule, which is a leukocytotrophic hormone
IL-4	Interleukin-4, a type of cytokine immune system signaling molecule that induces differentiation of naive helper T cells
iv	Intravenous

L	Liter
LDL-C	Low density lipoprotein-cholesterol
LOOH	Lipid peroxides
μM	Micromolar
μmol	Micromol
μL	Microliter
min.	Minute
mm	Millimeter
mM	Millimolar
mL	Milliliter
MUFA	Monounsaturated fatty acid
MW	Molecular weight
nm	Nanometer
nM	Nanomolar
nmol	Nanomol
$O_2^{\bullet-}$	Superoxide anion radical
$OH^{\bullet}$	Hydroxyl radical
Ox-LDL	Oxidized low density lipoprotein
PAD	Photodiode Array Detector
ppm	part per million
PUFA	Polyunsaturated fatty acid
$ROOH^{\bullet}$	Peroxyl radicals
ROS	Reactive oxygen species
RBO	Rice bran oil
SEM	Standard Error of Mean
SDS	Sodium dodecyl sulfate
SFA	Saturated fatty acid
TC	Total cholesterol
TG	Triacylglycerol (Triglycerides)

TNF-α	Tumor necrosis factor-alpha, a type of cytokine immune system signaling molecule that stimulates the acute phase reaction
VLDL-C	Very low density lipoprotein-cholesterol
v/v	Volume (millilier) by volume (milliliter)
w/v	Weight (gram) by volume (milliliter)

CHAPTER I

INTRODUCTION

1.1 Background and Rationale

Rice (*Oryza sativa* L.), one of the most important crops in the world, feeds a large part of the world's human population. According to the Food and Agriculture Organization (FAO), the global paddy production for 2008 was about 683 million tons (456 million tons, milled rice equivalent) (1). About 90% of the global paddy production was generated in Asian countries, especially Thailand, the leader of major rice exporter countries. The rice bran fraction generated during processing, accounts about 8% of total rice yield. Although the rice bran has a high nutraceutical value, it is normally used as animal feed. Edible oil and food supplements from rice bran contain several compounds with activities that can contribute to the prevention of chronic diseases such as cardiovascular diseases and cancer. For example, rice bran contains high levels of antioxidants such as tocopherols and tocotrienols. In addition, high levels of a mixture of unsaponifiable compounds referred to gamma-oryzanol (γ-OR) have been identified in rice bran (2, 3).

Gamma-oryzanol is a mixture of 10 distinct components that consist of triterpene derived compounds esterified to ferulic acid. Cycloartenyl ferulate, 24-methylenecyclo-artanyl ferulate, campesteryl ferulate, and sitosteryl ferulate are major components and account for 80% of γ-OR in rice bran oil (RBO)(3). Gamma-oryzanol is unique to RBO. However, the exact concentrations of γ-OR separated from RBO, vary substantially according to the rice cultivar, and the oil extraction and processing conditions (2, 4, 5).

Gamma-oryzanol has been shown to demonstrate both antioxidant (6-8) and hypocholesterolemic acitivities. The latter activity appears to be due to the inhibition of cholesterol absorption and synthesis (9-12). RBO and γ-OR have been reported to decrease serum cholesterol in animals and humans (9-11, 13-15) and can inhibit cholesterol oxidation *in vitro* (8). Ingestion of γ-OR also has been associated with a

significant reduction in aortic fatty streak formation in hamsters (11). In addition, it has been suggested that γ-OR modulated pituitary and gastric secretion, platelet aggregation, release of thyroid stimulating hormones, and immunomodulatory responses (16-18).

Lower cholesterol level in blood circulation may reduce the risk of cardiovascular diseases. In addition, high level of circulating low density lipoprotein (LDL), especially the oxidized LDL (Ox-LDL), also plays important roles in atherosclerosis development and increase cardiovascular diseases risk. It has been postulated that the Ox-LDL activates the immunologic response, and transforms inflammatory cells into foam cells and initiates the development of atherosclerotic plaques. Prevention of the Ox-LDL formation by the antioxidants is proposed to be helpful in decreasing the risk of cardiovascular diseases. Nevertheless, studies on the bioavailability of γ-OR are limited, especially with regards to digestibility and bioavailability of food containing γ-OR. In addition, it is still unclear how γ-OR can exert the hypocholesterolemic effect. Although it has been postulated that phytosterols and phytostanols could interfere with micellar solubility of cholesterol and lead to inhibition of cholesterol absorption (19-21), there were no such studies like those on γ-OR. Therefore, this study aimed to investigate the bioavailability of γ-OR and its mechanism on hypocholesterolemic activity *in vitro*. Effects of γ-OR on cholesterol micellarization and uptake by human intestinal cells, and the bioavailability of γ-OR were examined using *in vitro* simulated digestion coupled with human intestinal Caco-2 cell culture. Micellarization and intestinal cellular uptake of cholesterol with and without γ-OR were investigated. In addition, the inhibitory effect of γ-OR on the activity of HMG-CoA reductase, the step-limiting enzyme of cholesterol biosynthesis, was determined to investigate the mechanism of γ-OR on cholesterol biosynthesis. The antioxidant activities of γ-OR against LDL oxidation, compared with ferulic acid, and α-tocopherol were also observed in this study. Isolated LDL fractions from blood of healthy volunteers were prepared and the susceptibility tests of LDL to *in vitro* oxidation, with varies concentrations of γ-OR were assessed, compared with ferulic acid and α -tocopherol.

1.2 Research Questions

1.2.1 How does γ-OR micellarize into micelles during simulated digestion (bioaccessibility) and uptake into human intestinal cell (bioavailability)?

1.2.2 How does γ-OR affect the micellarization and uptake of cholesterol during simulated digestion and in synthetic micelles?

1.2.3 Do γ-OR and ferulic acid show inhibitory effect on HMG-CoA reductase activity?

1.2.4 How does γ-OR affect LDL oxidation compared with ferulic acid and α -tocopherol?

1.3 Objectives of the Study

1.3.1 To determine bioaccessibility and bioavailability of γ-OR by simulated digestion coupled with Caco-2 cells model

1.3.2 To determine effect of γ-OR on incorporation of cholesterol into synthetic micelles

1.3.3 To determine effect of γ-OR on incorporation of cholesterol into micelles generated during simulated digestion

1.3.4 To determine effect of γ-OR on uptake of cholesterol by simulated digestion couple with Caco-2 cells model

1.3.5 To determine inhibition activity of γ-OR and ferulic acid against HMG-CoA reductase, rate-limiting enzymes on cholesterol biosynthesis

1.3.6 To determine antioxidant activities of γ-OR against LDL oxidation, compared with ferulic acid and α-tocopherol

1.4 Hypotheses

1.4.1 Gamma-oryzanol is efficiently incorporated into micelles

1.4.2 Gamma-oryzanol can inhibit the micellarization of cholesterol during simulated digestion and in synthetic micelles lead to reduced cholesterol uptake by Caco-2 cells.

1.4.3 Both γ-OR and ferulic acid can inhibit HMG-CoA reductase activity.

1.4.4 Gamma-oryzanol can protect LDL from oxidation as same as ferulic acid and α-tocopherol.

1.5 Conceptual Framework

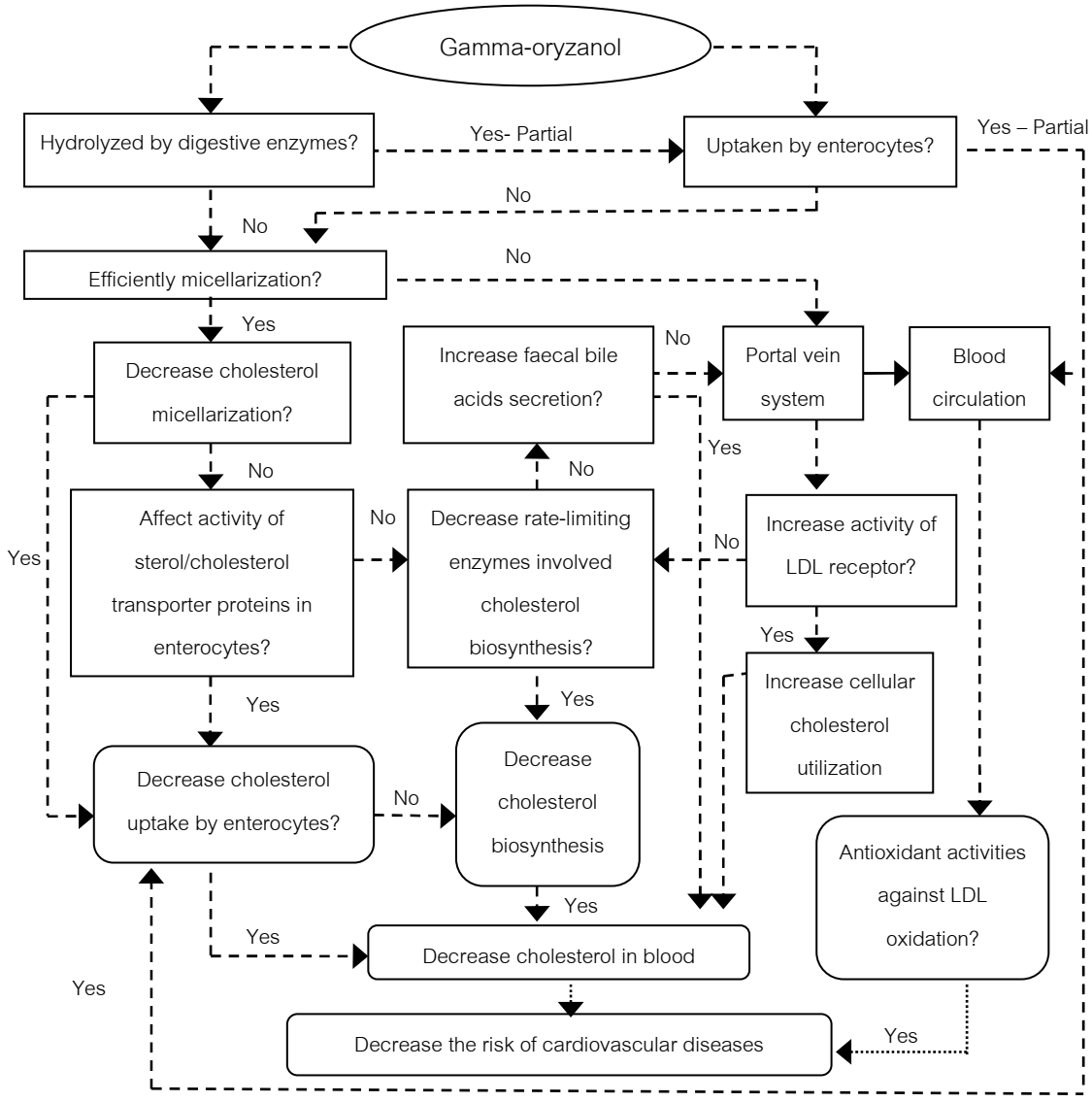


Figure 1.1 Conceptual framework of this study

CHAPTER II

LITERATURE REVIEW

Rice is “life” for many Asian countries, especially Thailand. Archeological evidences has shown that rice has been the staple food of Thai people from the ancient times (22). For Thais, life without rice is unimaginable. Furthermore, rice is one of the most important economic crops in Thailand. Thailand is one of the world biggest rice producers and exporters. In 2007, Thailand produced 18.3 million tons of milled rice equivalent and exported 8.5 million tons of those to the global market. Moreover, the amounts of rice production and export from Thailand tend to increase every year (23). In 2008, Thailand was the number one of the major global rice exporters and exported rice to the world about 10.0 million tons of milled rice equivalent. In addition, it was forecast that Thailand will keep the first rank of the major global rice exporters in 2009 (1).

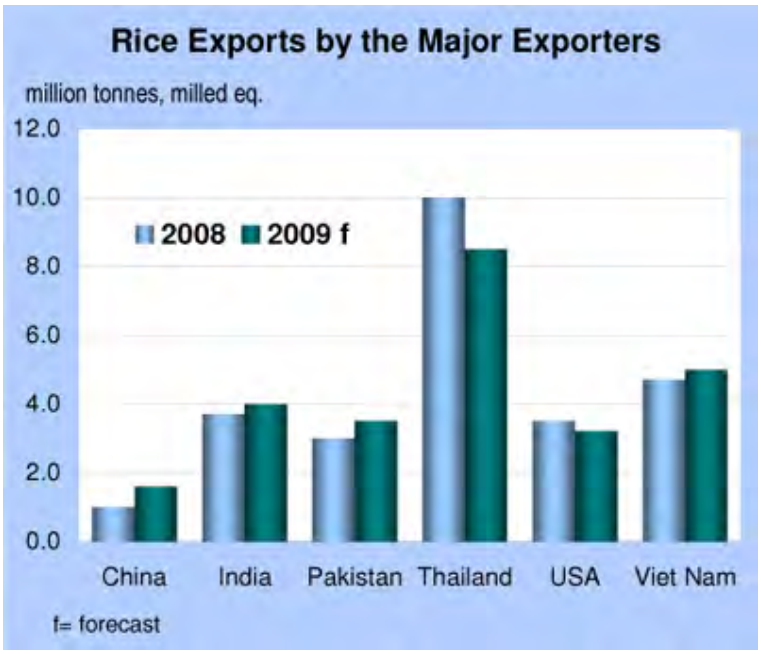


Figure 2.1 Rice exports by the major exporters; from Rice Market Monitor report (1)

High amount of milled rice equivalent exports means high production of rice by-product in the rice exporter countries. During rice milling process, rice bran fraction is generated and accounts about 8% of total rice yield. Although rice bran is a high

nutraceutical by-product from rice, it was normally used as animal feed and in edible oil production. Unfortunately, its nutraceutical value cannot be achieved as it should be.

2.1 Rice Bran and Rice Bran Oil

The rice grain is composed of three main layers. Those are the hull or husk, the rice bran and germ, and the inside kernel or endosperm as so called white rice (Figure 2.2). The rice hull or husk is a hard outer layer of rice grain. The hull is removed when the grain is milled. Underneath the hull is the rice bran and germ layer. The germ is the embryo of the grain that contained inside the kernel. The inside kernel of the rice grain under the aleurone layer is the endosperm or white rice. Rice bran fraction consists of the aleurone layer of the rice kernel and some part of the germ and endosperm. It is the rich source of proteins, lipids, vitamins, trace minerals, and bioactive compounds (24).

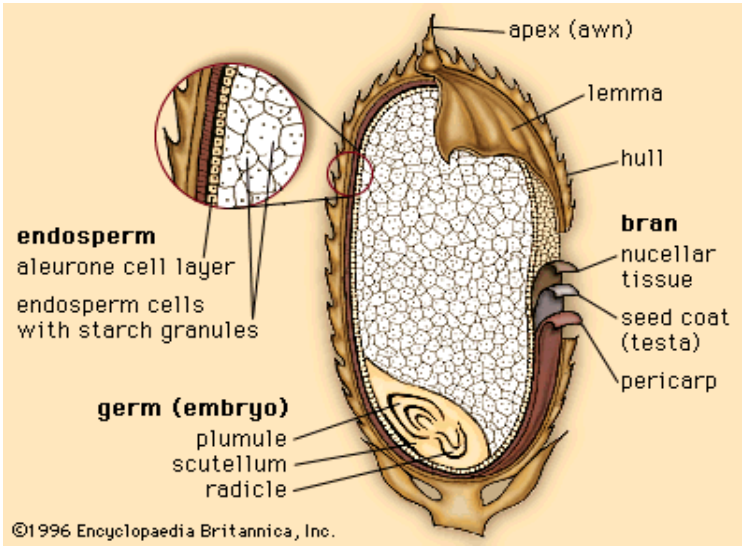


Figure 2.2 The outer layers and internal structures of a rice grain; Copyrights: © Encyclopædia Britannica, Inc.(25)

Rice bran fraction is a high nutraceutical by-product from rice milling process. It contains about 15-20% oil content by weight (24). Rice bran and its oil contain large concentrations of several compounds that could potentially prevent chronic diseases such as cardiovascular diseases and cancer. Unrefined rice bran oil contains high amounts of both mono- and poly- unsaturated fatty acids. It consists of approximately 20% saturated (palmitic acid; C16:0), 40% monounsaturated (oleic acid; C18:1), and

40% polyunsaturated fatty acids (linoleic acid; C18:2) (26). Moreover, it contains many antioxidants and phytochemicals such as tocopherols, tocotrienols, γ-OR, and β-sitosterol. Because of high natural antioxidants level in rice bran oil (RBO), it is also used as an ingredient in cosmetic and spa products. Moreover, extracted γ-OR from RBO also has been used as a food additive and for cosmetic purposes according to its antioxidant property.

2.2 Gamma-Oryzanol (γ-OR)

Gamma-oryzanol is a class of unsaponifiable lipids that is unique to RBO. It is a mixture of 10 components consisting of ferulic acid esterified to triterpene derived compounds (Figure 2.3). Cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, campesteryl ferulate, and sitosteryl ferulate are major components and account for 80% of γ-OR in RBO (3).

However, the exact level and composition of γ-OR content in RBO depend upon the rice cultivar, and the oil extraction and processing condition (4, 5). A wide range in the γ-OR content in RBO was reported. Refined RBO available in the United States contained total γ-OR levels of 115-787 mg/kg oil (5). Whereas, higher γ-OR levels of 10-25 g/kg oil were reported in the commercial RBO produced in India and Japan (27). In Thailand, commercial refined RBO by Thai Edible Oil Co.,Ltd., was claimed to contain γ-OR levels of at range 2-4 g/kg oil.

Gamma-oryzanol has been shown to have many biological activities such as hypocholesterolemic and antioxidant activities. It has been postulated in many studies that phytosterols and phytostanols could interfere with micellar solubility of cholesterol and lead to inhibition of cholesterol absorption (19-21). Therefore, it is possible that γ-OR can lower blood cholesterol because of its chemical structure similarly to cholesterol (Figure 2.3). However, it is still unclear how γ-OR can exert the hypocholesterolemic effect.

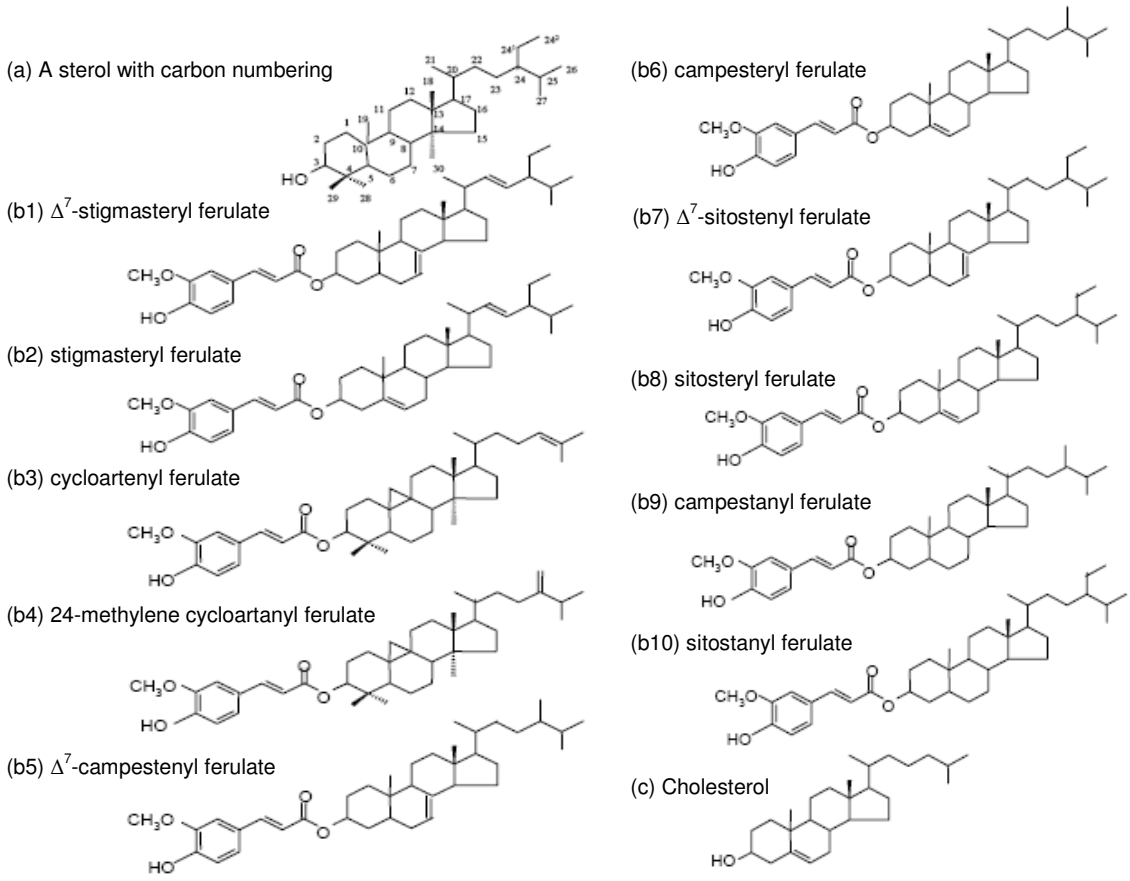


Figure 2.3 Chemical structures of (a) sterol and carbon numbering, (b1)-(b10) identified components of γ-OR by Xu and Godber (3), and (c) cholesterol; photo adapted from Huang (28)

2.2.1 Gamma-Oryzanol and Hypocholesterolemic Activity

RBO and γ-OR have been reported to decrease serum cholesterol in many animals and human studies (9-11, 13-15). Fukushima *et al.* found that cecectomized rats fed rice bran had significantly lower concentrations of serum total cholesterol, serum triacylglycerol and the liver cholesterol than control (fed cellulose) rats (9). Rong *et al.* found that γ-OR-treated hamsters exhibited a 25% reduction in percent cholesterol absorption and had a 67% lower aortic fatty streak formation, compared with control animals (11).

Most *et al.* investigated the effects of defatted rice bran and RBO in an average American diet on blood lipids in moderately hypercholesterolemic human subjects (10).

The results from the study showed significantly lower blood total cholesterol in subjects consuming the diet containing RBO compared to control diet. Moreover, consumption of the diet containing RBO decreased low density lipoprotein-cholesterol (LDL-C) by 7%, whereas high density lipoprotein-cholesterol (HDL-C) was unchanged. Berger *et al.* assessed the cholesterol lowering effects of RBO with low and high amounts of γ-OR (0.05 g/day and 0.8 g/day) in mildly hypercholesterolemic men (13). They found that ingestion of the two types of RBO similarly affected several cholesterol parameters. However, consumption of RBO containing low and high γ-OR for 4 weeks at approximately 50% of total fat intake improved the lipoprotein pattern by lowering total plasma cholesterol, LDL-C and LDL-C/HDL-C ratio by 6.3%, 10.5% and 18.9%, respectively.

Effect of RBO and its components on lipid metabolism was summarized by Cicero and Gaddi (16) (Table 2.1). However, it has been speculated that the hypocholesterolemic effect of RBO is attributed to its specific components, γ-OR, not its fatty acid composition. Nevertheless, the mechanism how γ-OR reduces blood cholesterol is still not completely investigated.

It was believed that plant sterols and stanols (phytosterols) can inhibit cholesterol absorption due to the similar structure to cholesterol that affecting; 1) micellar solubilization of cholesterol; 2) co-crystallization of cholesterol and phytosterols from mixed micelles; 3) the activities of lipases, esterases, and other enzymes involved in the sterol absorption process; and 4) the activity of brush border transport (29). However, the effect of phytosterols on cholesterol biosynthesis is one topic that should be concerned in the hypocholesterolemic machanism.

The chemical structure of γ-OR components are similar to cholesterol (Figure 2.3). Therefore, it is possible that hypocholesterolemic activity of γ-OR may result from both inhibition of cholesterol absorption and cholesterol biosynthesis mechanisms as other phytosterols that described above. In one study, it was found that preincubation of γ-OR and cholesterol during micelles preparation for six hours before treated to cells significantly decreased the cholesterol uptake *in vitro* cell model (28). It was suggested

that γ-OR may decrease cellular cholesterol uptake by lowering cholesterol micellarization during digestion.

Table 2.1 Effect of rice bran oil and its main components on lipid metabolism (16)

Animal species	Rice bran oil component	Dose, Administration route	Pharmacological effect	Reference
rats	γ-oryzanol	0.5% – 2% of dietary fat, oral	↓ TC, LDL-C, HDL-C, VLDL-C ↓ liver cholesterol esters and triglycerides	Shinomiya *et al.* (1983)
rats	γ-oryzanol	10 mg/ kg, iv	↓ TC, TG, non-esterified fatty acids and free cholesterol	Sakamoto *et al.* (1987)
rats	Cycloartenyl ferulate	10 mg/ kg, iv	↓ TC, TG, non-esterified fatty acids and free cholesterol	Sakamoto *et al.* (1987)
rats	rice bran oil	10% of dietary fat , oral	↓ TC, LDL-C, VLDL-C, TG, ↑HDL-C ↓ liver cholesterol and triglycerides ↑ faecal bile acids and neutral sterols	Sharma and Rukmini (1986; 1987)
rats	rice bran oil	10% of dietary fat, oral	↓ TC, LDL-C, VLDL-C, ↑HDL-C ↓ liver cholesterol and triglycerides	Seetharamaiah and Chandrasekhara (1988; 1989)
rats	rice bran oil	5%-20% of dietary fat, oral	↓ TC, LDL-C, VLDL-C, TG, ↑ HDL-C	Purushothama *et al.* (1995)
rats	rice bran oil + safflower oil	10% of dietary fat, oral	↓ TC, LDL-C, TG, ↑HDL-C ↓ liver cholesterol and triglycerides ↑ faecal neutral sterols and bile acids	Sunitha *et al.* (1997)
rabbits	γ-oryzanol	1% of dietary fat, oral	↓ oleate incorporation into cholesteryl ester by macrophages	Hiramatsu *et al.* (1990)
hamsters	γ-oryzanol	1% of dietary fat, oral	↓ TC, LDL-C, VLDL-C, TG, HDL-C ↓ non-HDL-C/HDL-C ratio ↓cholesterol absorption and aortic fatty streak formation	Rong *et al.* (1997)
monkeys	rice bran oil	20%- 25% of dietary fat, oral	↓ TC, LDL-C, apolipoprotein B	Nicolosi *et al.* (1991)
humans	γ-oryzanol	300 mg, oral	↓ TC, LDL-C, TG, ↑HDL-C	Ishihara *et al.* (1982)
humans	γ-oryzanol	300 mg, oral	↓ TC, LDL-C, ↑HDL-C	Yoshino *et al.* (1989)
humans	γ-oryzanol	100 mg x 3, oral	↓ TC, LDL-C, apolipoprotein B	Sasaki *et al.* (1990)
humans	rice bran oil	42 g, oral	↓ TC, LDL-C	Suzuki and Oshima (1970)
humans	rice bran oil	50 g, oral	↓ TC, LDL-C, ↑HDL-C	Tsuji *et al.* (1989)
humans	rice bran oil	50 g, oral	↓ TC, LDL-C, TG	Raghuram *et al.* (1989)

** iv, intravenous; TC, total cholesterol; LDL-C, low density lipoprotein-cholesterol; VLDL-C, very low density lipoprotein-cholesterol; HDL-C, high density lipoprotein-cholesterol; TG, triacylglycerols*

2.2.2 Gamma-Oryzanol and Antioxidant Activity

Gamma-oryzanol has been reported to exhibit antioxidant properties and can inhibit cholesterol oxidation *in vitro* (4, 7, 8, 30). Xu *et al.* investigated the antioxidant activity of γ-OR components from rice bran against cholesterol oxidation induced by 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH) (8). They found that extracted γ-OR components (cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, and campesteryl ferulate) exhibited significant antioxidant activity in the inhibition of cholesterol oxidation. The highest antioxidant activity was for 24-methylenecycloartanyl ferulate, and all three γ-OR components had activities higher than that of any of the tested vitamin E components (α-tocopherol, α-tocotrienol, γ-tocopherol, and γ-tocotrienol).

An examination of the antioxidant properties of rice bran varieties was recently reported (4). Analyses included the measurement of total phenolic content, antioxidant activity in linoleic acid system, reducing power, metal chelating ability, scavenging capacity by 2,2'-diphynyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) and 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) radical, and conjugated dienes. The results supported the conclusion that the unique mixture of tocols and oryzanol in rice bran is responsible for its potent antioxidative properties.

Gamma-oryzanol inhibited lipid soluble organic radicals at concentration of 50-100 μM in one study (30). However, this effect was observed only when γ-OR was incorporated within liposomes, suggesting that γ-OR cannot spontaneously localize within the lipid phase. In addition, γ-OR was not scavenger of $OH^{\bullet}$ nor $O_2^{\bullet-}$ in this study. On the other hand, preparation of γ-OR in liposomes or micelles seems to be important for the *in vitro* studies of γ-OR.

2.2.3 Other Bioactivities of γ-OR

RBO has been reported to modulate of pituitary and gastric secretion, release of thyroid stimulating hormones, platelet aggregation, and immunomodulatory responses (16-18). In an animal study, RBO has been shown to have antiallergenic property by

enhancing B-lymphocyte proliferation and IL-2 or TNF-α, and decreased IL-4 and IgE levels. In the same study, the role of γ-OR on the immune system was also determined. Gamma-oryzanol was able to increase TNF-α level, but no effect on B-lymphocyte proliferation or IL-4 expression. The results suggested that γ-OR is not responsible for the overall immnunostimulation effect seen for RBO, even though it may modulate the immune system (18).

2.2.4 Side Effects and Toxicology of γ-OR

Phytosterols are assumed to undergo oxidation reactions similar to those of cholesterol. During oxidation process, the unstable free radicals are generated. These free radicals can cause damages to cells. However, phytosterols and their fatty acid esters are very stable compounds. Even under severe conditions, such as during deep-frying, sterol oxidation products are formed at low (ppm) concentrations. Furthermore, phytosterols do not exhibit *in vitro* mutagenic activity or subchronic toxicity in animals (29). In addition, there is no evidence that γ-OR adversely affects reproductive performance or exhibits mutagenic and carcinogenic effects *in vitro* or *in vivo* (28).

However, phytosterols can interfere with the absorption of carotenoids leading to a decrease in carotenoids levels in the blood (31). Nevertheless, the mechanisms responsible for the interaction between γ-OR and carotenoids have not been investigated.

2.2.5 Quantification and Identification of γ-OR

The most commonly used techniques for the determination of γ-OR in RBO are high performance liquid chromatography (HPLC) and UV spectrophotometry. The HPLC methods are generally applied to identify the chemical composition of γ-OR contents in rice bran. The UV spectrophotometric methods are preferred to determine total content of γ-OR because it is more simple, rapid and inexpensive than HPLC (32). However, the interference of the oil matrix may produce incorrect results, particularly for samples with low concentrations of γ-OR and measuring the absorbance at a single wavelength.

Three spectrophotometric methods for the determination of γ-OR in RBO were compared in a study (32). They found that at fixed wavelength, the results were more accurate when using isopropanol (measured at λ_{max} = 327 nm), compared to *n*-heptane (measured at λ_{max} = 314 nm) to dilute oil samples. However, to obtain accurate results in oil with a low content of γ-OR, it is necessary to perform the spectrophotometric analysis using second-derivative UV spectrum (^{2}D 330.365) to eliminate interference by the oil matrix.

Determination and identification of individual γ-OR components in RBO generally involves extraction step and followed by HPLC. Those extraction methods include liquid-liquid phase extraction, solid phase extraction, superficial fluid extraction and direct solvent extraction (33-35).

Direct solvent extraction method has been most commonly used because it does not require special instrumentation (36). This extraction method generally involves repeated extractions of RBO with hexane. More polar solvents than hexane such as isopropanol, isopropanol: hexane (1:1; v/v), or methanol also have been used for direct solvent extraction method.

A simple one-step equilibrium extraction procedure coupled with reverse-phase HPLC for γ-OR analysis was reported by Chen and Bergman (36). They used the one minute equilibrium extraction at a 1:60 (w/v) ratio of rice bran to solvent and found that methanol and isopropanol were superior extraction solvents compared to hexane. They suggested that extraction of rice bran with methanol and reverse-phase HPLC with mobile phase gradient containing acetonitrile, methanol, isopropanol and 1% aqueous acetic acid was optimal for γ-OR quantification.

Identification of γ-OR by various HPLC methods showed that γ-OR is a mixture of several components. Xu and Godber extracted γ-OR from RBO using low-pressure chromatography and preparative normal-phase HPLC to reduce interfering substances and obtain high-purity γ-OR (3). Concentrated γ-OR was further analyzed using reverse-phase HPLC. Ten fractions of γ-OR were separated and individually collected. Chemical

structures of individual fraction were further identified using gas chromatography-mass spectrometry (GC-MS) technique. The 10 components of γ-OR were identified as Δ^7-stigmastenyl ferulate, stigmasteryl ferulate, cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, Δ^7-campestenyl ferulate, campesteryl ferulate, Δ^7-sitostenyl ferulate, sitosteryl ferulate, campestanyl ferulate, and sitostanyl ferulate, respectively (Figure 2.3).

2.3 Bioavailability of γ-OR

It has been known that phytosterols has low solubility and low absorption potential by enterocytes (29). Phytosterols must be taken in high dosage to achieve a reduction in cholesterol level. One of the goals of the food and pharmaceutical industries is to develop phytosterols products with higher sterols bioavailability, higher bioefficacy as hypocholesterolemic agents, with smaller dosage.

Although γ-OR has been used in many countries as a food additive and a food supplement because of its hypocholesterolemic and antioxidant activities, there are not many studies exploring the bioavailability of γ-OR. However, γ-OR is postulated to have the same absorption activity in the small intestine as other phytosterols. Understanding the bioavailability and hypocholesterolemic mechanism of γ-OR is useful for future γ-OR products development.

2.3.1 *In vitro* Studies

The results of *in vitro* digestibility studies of γ-OR showed that the ester bond of some γ-OR components, especially campesteryl ferulate and sitosteryl ferulate, was cleaved by cholesterol esterase and produced triterpene alcohols or sterols and ferulic acid, which were further degraded. However, cycloartenyl ferulate and 24-methylenecycloartanyl ferulate were not hydrolyzed by cholesterol esterase. Lipase and phospholipase A_2 had no efficiency to hydrolyze γ-OR (28, 37). However, γ-OR was not hydrolyzed in the stomach because sterols or triterpene alcohols were not found after peptic digestion (28).

The uptake of γ-OR (in crystalline or in synthetic micellar solution) into intestinal cells was determined after *in vitro* digestion using a human intestinal cell line (C2BBe1 cells; ATCC CRL-2102) (28). Intact γ-OR was not detected in cells after 2-hours incubation of digested γ-OR. Nevertheless, longer incubation time of digested γ-OR was not further conducted in the study. However, the digestibility and bioavailabilty of γ-OR in the test meal undergo simulated oral, gastric and small intestinal digestions were not conducted in the studies.

2.3.2 Animal Studies

In 1983, Fujiwara *et al.* studied the absorption and metabolism of γ-OR in rats (38). Rats were orally administered γ-OR-^{14}C (50 mg/kg) in their study. The result of *in situ* absorption experiment at the mid-ileal portion of the intestines suggested that γ-OR was poorly absorbed. Almost 90% of the dosage remained in the luminal fluid with more than 95% intact form and only 0.2% found in mesenteric vein as intact esters. Only 0.3% of the dose was transported into the thoracic duct as intact γ-OR. It was suggested that the absorbed γ-OR from oral administration mainly went through the portal vein system instead of lymph.

After 72 hours of oral γ-OR-^{14}C administration, 84.5% and 9.8% of radioactivity was found in feces and in urine, respectively. Intact γ-OR was not found in urine but the major metabolites were detected such as ferulic acid, dihydroferulic acid, *m*-hydroxyphenylpropionic acid, *m*-coumaric acid, *m*-hydroxyhippuric acid, hippuric acid, and their conjugated forms with glucuronide or sulfate (Figure 2.4). The ester linkage of γ-OR was partly hydrolyzed in the intestine during absorption. However, the digestibility of γ-OR in the stomach, duodenum, and jejunum were not reported.

2.3.3 Human Studies

It was mentioned by Umehara *et al.* that γ-OR is mainly metabolized to ferulic acid in animals and humans (39). Very few studies on bioavailability of γ-OR in human were reported. They also mentioned about a study by Odomi *et al.* which reported that the peak plasma concentrations of γ-OR and ferulic acid were 37.6 and 36.6 ng/mL,

respectively when administered a single oral of γ-OR at 300 mg to humans. In addition, ferulic acid was excreted in urine at 2.4 -2.8% of the dosage/day, but intact γ-OR was not detected (39).

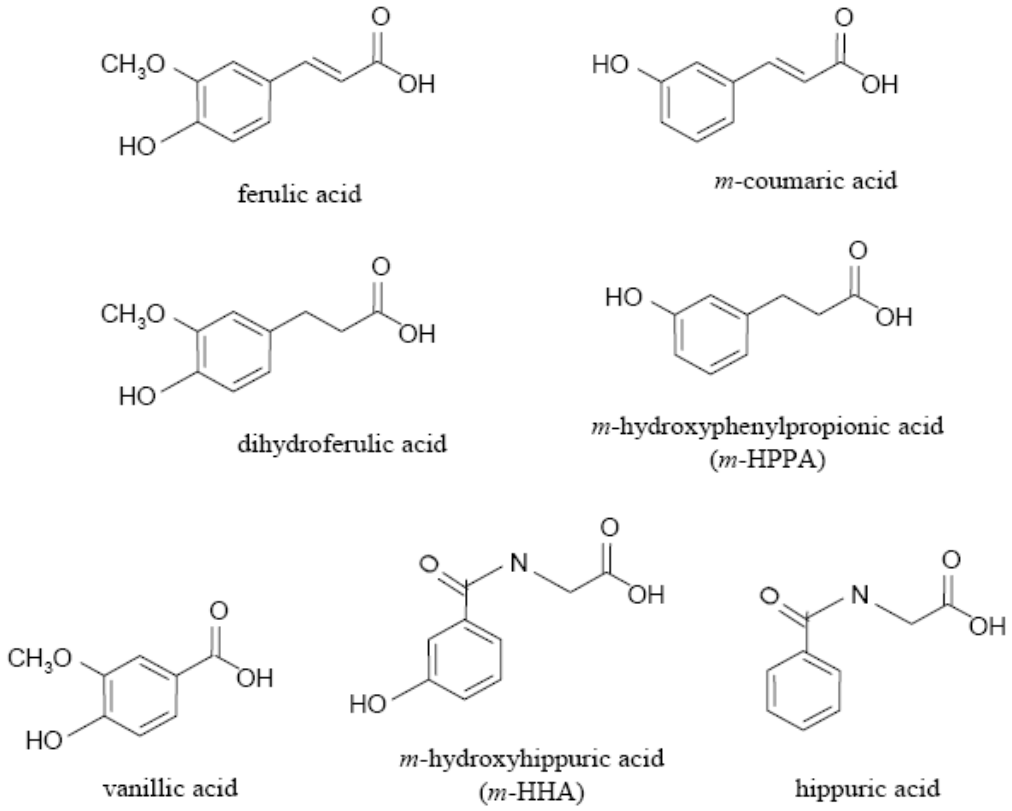


Figure 2.4 Chemical structures of ferulic acid and its urinary metabolites; photo adapted from Huang (28)

2.4 Caco-2 Cells and Bioavailability Studies

Cultured cells have been used to investigate antioxidant activities, cholesterol absorption, and bioavailability in many studies with the varieties of substances (28, 40-44). Selection of a cell line for investigation depends on the specific functions to be studied. In addition, characteristics of the cell line are needed to be considered such as types, species, growth characteristics, availability, stability, validity, and phenotypic expression. Those characteristics of cell line can be achieved by selecting the reliable source of cell culture. The American Type Culture Collection (ATCC) is the most reliable resource that provides the high quality cell lines.

The uptake of γ-OR (in crystalline or in synthetic micellar solution) into cells after *in vitro* digestions was conducted in a study using a human large intestinal cell line, C2BBe1 cells (ATCC CRL-2102) (28). However, Caco-2 cells (ATCC HTB-37) model is the gold standard *in vitro* model of intestinal absorption studies.

The parent Caco-2 cell line (HTB-37), a human colon adenocarcinoma cell line, is available from ATCC. It was normally used in the *in vitro* model to study the regulation of processes associated with the apical uptake, metabolism, and transepithelial transport of diverse nutrients and other dietary components. Morphology and the biochemical characteristics of differentiated Caco-2 cells are similar to enterocytes. These cells spontaneously differentiate into enterocyte-like cells when monolayer reach confluency and are maintained under conventional conditions. During the early phases of differentiation, the cells express both colonocyte- and enterocyte- specific proteins. After approximately 2 weeks, the differentiated monolayer is characterized by tight junctions between cells, apical brush border surface enriched with hydrolytic enzymes, and expression of many transport proteins as enterocytes in small intestine.

However, some characteristics of differentiated Caco-2 cells differ from those of small intestinal enterocytes such as origin of cells, and genetically and phenotypically heterogeneous characters. In addition, Caco-2 cells show more colonic characteristic of transepithelial resistance than small intestinal epithelium. Furthermore, Caco-2 cells use the glycerol 3-phosphate pathway for the synthesis of triacylglycerols instead of the monoacylglycerol pathway. Some of these differences can be limited by standardization of growth and maintenance procedures, and the design of studies using this cell line.

Strictly controlled factors such as source of cells, range of passage numbers used for experiments, degree of maturation of cells at the time of investigations, and condition used in the studies, can minimize genetic and phenotypic drift of Caco-2 cells. For example, Caco-2 cells should be used between passages 20-45 for investigations. Moreover, the cells require a minimum of 10-12 days after monolayer reaches confluency to mature to the enterocyte-like state and 21-25 days for effective synthesis

and secretion of lipoproteins. The strictly controlled experiments facilitate comparison of results within and among laboratories (45).

2.5 Micelles and γ-OR Digestion

In digestion, mobilization and transport of cholesterol, dietary lipids and lipophilic compounds, micelles formation plays a very important role. Gamma-oryzanol is a lipophilic substance due to its chemical structure similarly to cholesterol. Therefore, micelles formation is also essential for γ-OR digestion.

Digestibility of γ-OR (in crystalline or in synthetic micellar solution) after *in vitro* peptic and pancreatic digestion was conducted in a study (28). It was found that γ-OR in micellar solution was degraded more than in the crystalline form. The reason may be relative to increased accessibility of micellar γ-OR to the digestive enzymes. This was proving that micelles formation is necessary for lipophilic compounds digestion, including γ-OR.

Dietary lipids and their digestion products must be incorporated into micelles with the help of amphipathic compounds such as bile salts, phospholipids and proteins before being transported across the intestinal cell surface. Amphipathic biomolecules contain both polar and nonpolar regions, so the mixed micelles can solubilized in aqueous phase in lumen.

Bile salts are synthesized from cholesterol in liver and stored in the gallbladder for release into the duodenum. The formation of mixed micelles increases the action of pancreatic lipase to convert triacylglycerols into monoacylglycerols, diacylglycerols, free fatty acids and glycerols, which can be absorbed into intestinal epithelial cells. Within these cells, triacylglycerols will be resynthesized from esterification of monoacylglycerols and fatty acids and incorporated with dietary cholesterol, and apolipoprotein B-48/ B-100 into chylomicrons, one type of lipoproteins in blood. Lipoproteins are responsible for transporting phospholipids, triacylglycerols, cholesterols, cholesteryl esters, lipophilic vitamins, and other lipophilic compounds between organs. Chylomicrons deliver newly absorbed lipids to tissues in the body

through the lymphatic system. Uptake of chylomicron remnants by liver leads to transfer of lipids to secreted very low density lipoproteins (VLDL) with subsequent re-distribution of the lipids and apolipoproteins among LDL and HDL. On the other hand, lipoproteins synthesis and transport are important for lipids and cholesterol metabolism in the body. The synthesis and transport of lipoproteins were shown in Figure 2.5.

2.6 Cholesterol and Lipoproteins Metabolism

Circulating cholesterol in body is either obtained from diet or synthesized in a variety of tissues, including the liver, adrenal cortex, skin, intestine, testicles and aorta. Both dietary cholesterol and that synthesized *de novo* are transported through the circulation of lipoproteins and store in cells in cholesteryl esters form. Dietary cholesterol is transported from the small intestine to the liver within chylomicrons. Cholesterol that exceeds hepatic needs, both synthesized by the liver and any dietary cholesterol in the liver, is transported in blood circulation within LDL. Cholesterol in peripheral tissues can be extracted by HDL and esterified by lecithin: cholesterol acyl transferase (LCAT), transferred to VLDL and LDL and then return to the liver via LDL circulation called as reverse cholesterol transport (Figure 2.5). Excess cholesterol can be excreted in bile as free cholesterol or as bile salts following conversion to bile acids in the liver.

Cholesterol homeostasis in the body is maintained primarily by controlling the level of *de novo* synthesis. The rate-limiting step of cholesterol biosynthesis is the reduction of HMG-CoA to mevalonate by HMG-CoA reductase enzyme which requires NADPH as a cofactor. Then mevalonate undergoes repeated phosphorylations, polymerization, cyclization and converted to various isoprene compounds as intermediate precursors to be cholesterol. Inhibition of cholesterol biosynthesis by inhibitors such as statins, which mimic mevalonate structure, will block the active site of HMG-CoA reductase. This mechanism of HMG-CoA reductase inhibitor is one principle to lower blood cholesterol level (Figure 2.6).

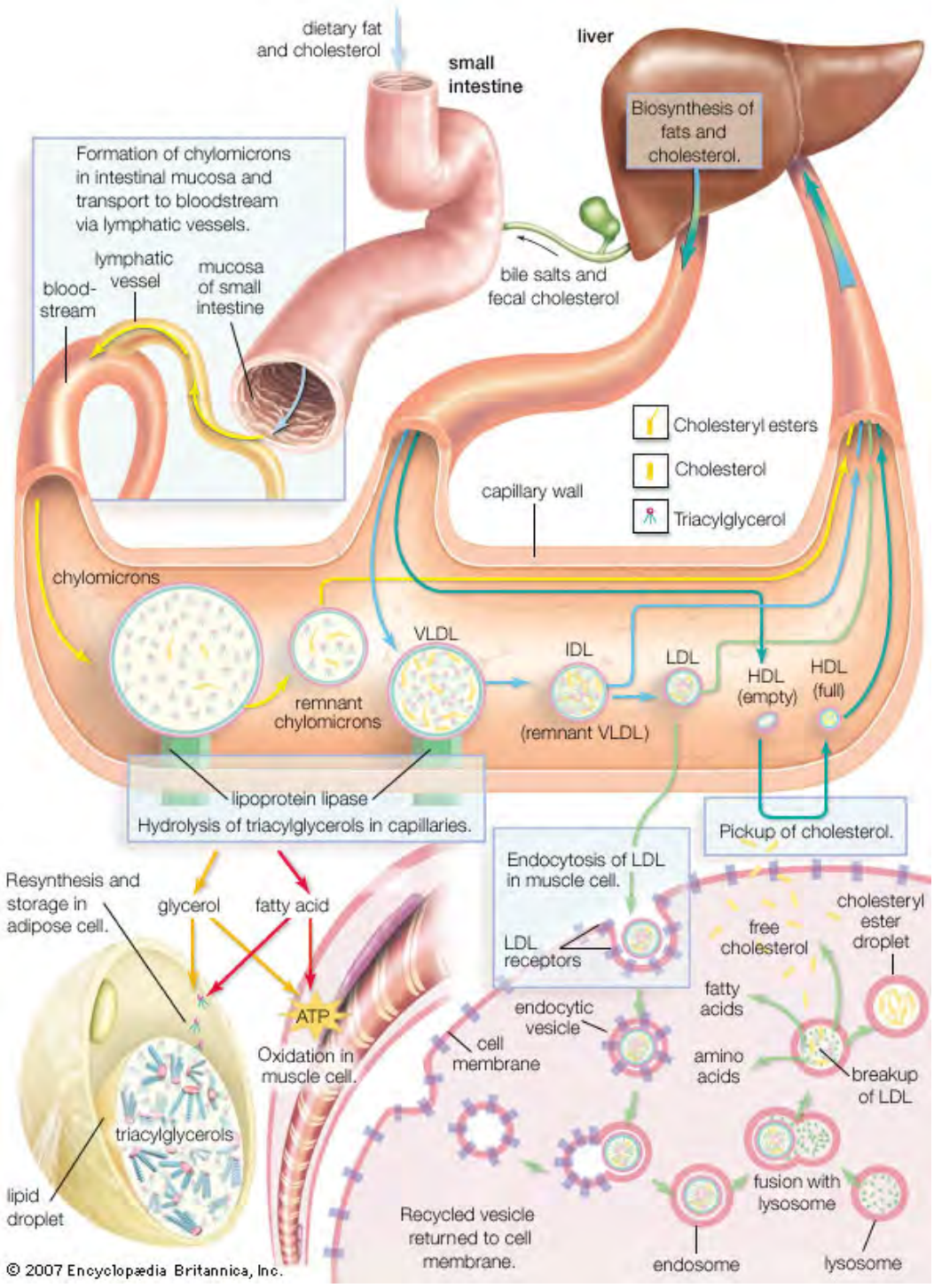


Figure 2.5 Synthesis and transport of lipoproteins; Copyrights: © Encyclopædia Britannica, Inc.(46)

It has been reported that β-sitosterol can decrease HMG-CoA reductase activity as well as its mass and mRNA levels *in vitro*. The activity of HMG-CoA reductase in ileac mucosal cells and hepatocytes was also significantly lower in sitosterol-fed rats

compared to control animals (47). However, effect of γ-OR on HMG-CoA reductase activity is still unclear.

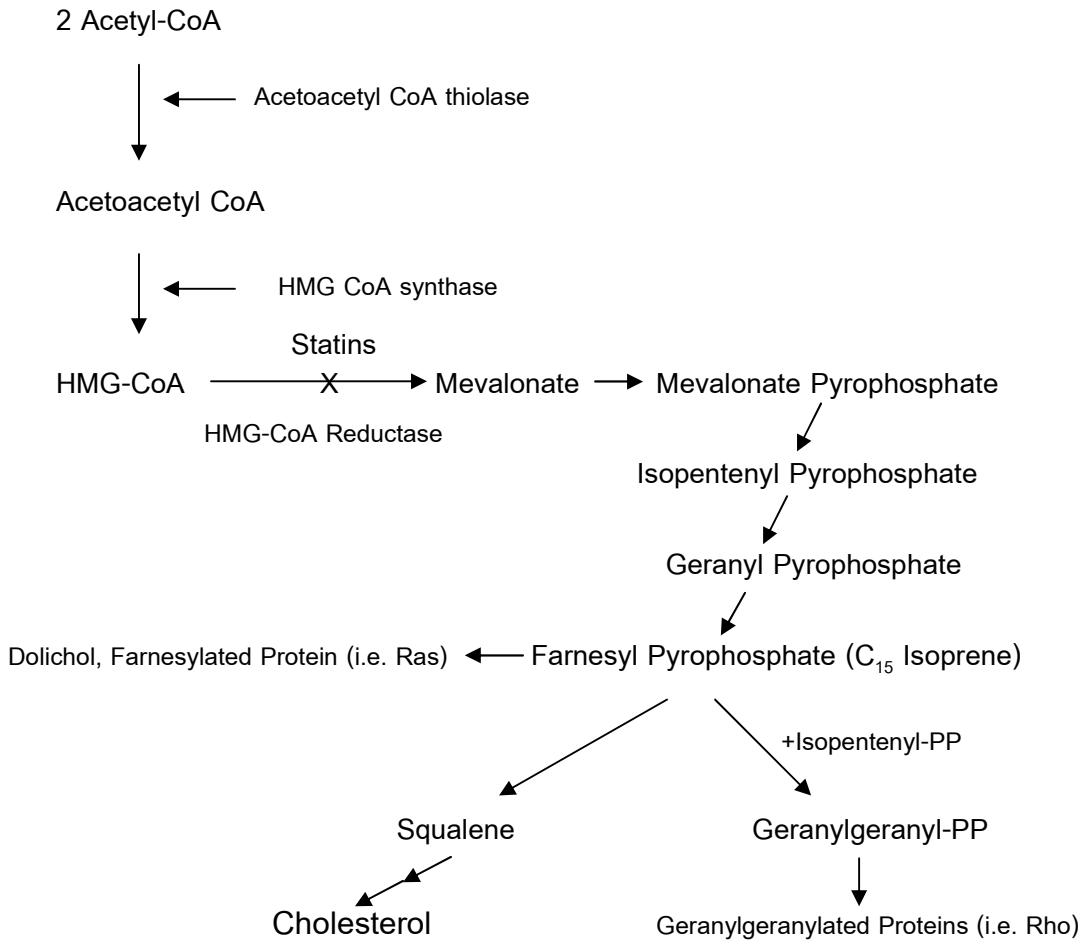


Figure 2.6 Cholesterol biosynthesis pathways, adapted from Brown and Goldstein (48)

2.7 Related Transport Proteins in Sterols Absorption

Intestinal absorption of sterols regulated with many transporters. It is speculated that ABCG5 (ATP binding cassette) and ABCG8 function as sterol transporters, facilitating the efflux of plant and animal sterols taken up by enterocytes into the lumen of the gut. ABCA1, another sterol transporter has been implicated in facilitating the efflux of cholesterol at the basolateral membrane of intestinal cells. Niemann-Pick C1-like1 (NPC1L1) was recently described and found to be critical for the normal absorption of cholesterol and phytosterols (49-52). Further researches on the expression of these transporters and their roles in the transport of phytosterols and γ-OR are still needed.

2.8 LDL Oxidation

High level of blood cholesterol, particular LDL-associated cholesterol, is demonstrated to be one of the main risk factors of atherosclerosis and cardiovascular diseases. Normally the uptake of cholesterol by the classical LDL receptor pathway cannot result in appreciable cholesterol accumulation because the LDL receptor is subject to feedback accumulation by the intracellular cholesterol content. When the initially oxidation of LDL occurs, minimally modified LDL (MM-LDL) with mild lipid peroxidation, can be uptake by the classical LDL receptor. However, MM-LDL can induce leukocyte-endothelial adhesion and secretion of monocyte chemotactic protein-1 (MCP-1) and macrophage colony-stimulating factor (M-CSF) by the endothelium. This results in monocyte binding and recruitment to the endothelium and subsequent migration into the subendothelial space, where M-CSF promotes their differentiation into tissue macrophages. Macrophages in turn can modify MM-LDL into a more oxidized form. Unfortunately, the more oxidized LDL (Ox-LDL) is no longer recognized by LDL receptor, so it is taken up by the scavenger receptor on the monocyte-macrophages instead. Because the scavenger receptor mechanism is not regulated by the intracellular cholesterol content, it results in cholesterol accumulation within the macrophages and contributes to triggering inflammation, transformation of inflammatory cells to be foam cells, and develop atherosclerotic plagues which in turn increases the damage of endothelium (Figure 2.7). In addition, the oxidizability of LDL also depends on its size. It has been shown that small dense LDL is more susceptible to oxidation than normal-sized LDL (53).

2.8.1 Oxidative Modification of LDL

Oxidatively LDL modification can occur even in a cell-free system or a cellular system. In a cell-free system, the transition metals such as iron and copper can oxidize LDL by binding with LDL and produce rapid lipid peroxidation. In the cellular system, all the major cells of the arterial wall such as endothelial cells, smooth muscle cells, and monocyte-macrophages involve in LDL modification. Superoxide anion, mediated by smooth muscle cells and phagocytes can promote oxidation of LDL lipids. The

membrane-associated NADPH oxidase of activated phagocytes such as human neutrophils, and monocytes, also oxidize LDL via a pathway which is inhibited by superoxide dismutase and metal chelators. In addition, autooxidation of thiol in the presence of metal ions that occur in arterial smooth muscle cells, cause thiyl radicals and superoxide which promote LDL oxidation. Certain cellular enzymes, such as 15-lipoxygenase and phospholipase A_2 have also been shown to stimulate LDL oxidation *in vitro*. The heme protein, myeloperoxidase, secreted by activated phagocytes, may act as a catalyst in lipoproteins oxidation. Nitric oxide and peroxynitrite, produced by endothelial cells and macrophages, are also relevant to LDL oxidation. Therefore, there are numerous mechanisms that cause oxidatively LDL modification (53).

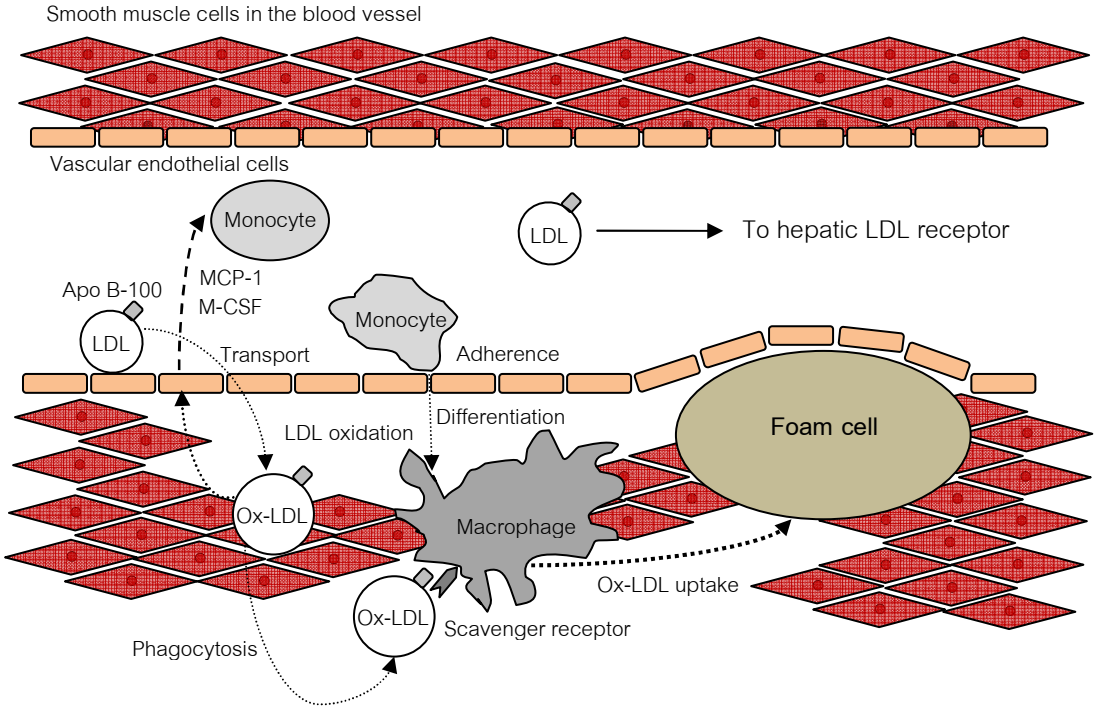


Figure 2.7 LDL, oxidized LDL and atherosclerosis formation

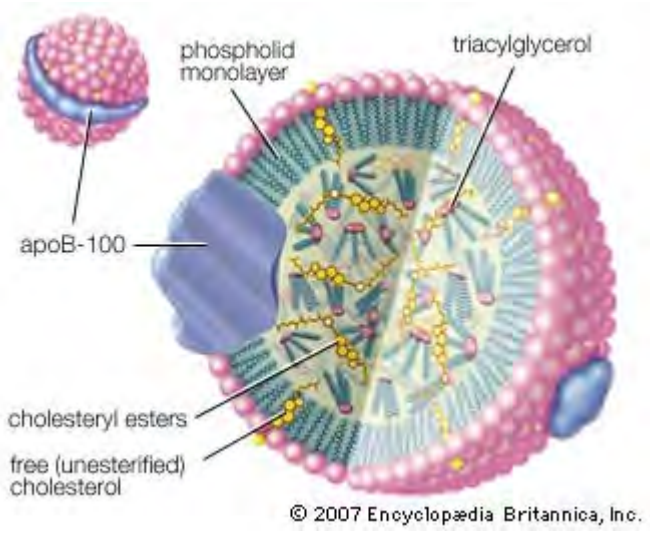


Figure 2.8 Cutaway view of a low density lipoprotein (LDL) complex; Copyrights: © Encyclopædia Britannica, Inc.(54)

LDL is defined as the population of lipoproteins that have a density range of 1.019 – 1.063 g/mL. LDL fraction can be isolated by ultracentrifugation. Cutaway view of a LDL particle is shown in Figure 2.8. Each LDL particle has a central lipophilic core that contains about 1,600 molecules of cholesteryl ester and 170 molecules of triacylglycerols. This core is surrounded by a monolayer of about 700 phospholipid molecules, consisting mainly of lecithin and small amounts of sphingomyelin and lysolecithin and 600 molecules of free cholesterol. Embedded in the outer layer is a large protein, apolipoprotein (apo) B-100, consisting of 4,536 amino acid residues. In an LDL molecule, about 2,700 molecules of total fatty acids bound in different classes. Half of these fatty acids are polyunsaturated fatty acids (PUFAs), mainly linoleic acid. Variations in PUFAs content and several antioxidants content such as α-tocopherol in LDL particle contribute to the difference in the oxidation behavior of different LDL samples (53).

Oxidation of LDL is a free radical-mediated process, resulting in several structural changes. The peroxidation of PUFAs in LDL is a common initiating step. Oxidation of LDL is initiated by reactive oxygen species that abstract an $\dot{H}$ from a double bond in PUFA, followed by molecular rearrangement and the formation of conjugated double bonds in the molecule referred to 'conjugated dienes'. During this initiation

phase of LDL oxidation, the rate of oxidation is suppressed by the presence of endogenous antioxidants within the LDL particle, which resulting in the lag phase of oxidation. The lag phase is followed by the rapid propagation phase that occurs when the antioxidants are depleted and the abstraction of another $H^{\bullet}$ by a PUFA-peroxyl radical ($LOO^{\bullet}$) from another PUFA, resulting in the formation of lipid peroxides. The propagation phase is followed by the decomposition or degradation phase, which the cleavage of double bonds and the formation of aldehydes occur. The major aldehydes produced in this phase include malondialdehyde (MDA), 4-hydroxynonenal (HNE), and hexanal, which can cross-link with amino groups on apo B-100 (53).

During the oxidation of LDL, changes in the protein moiety of the LDL particle also occur. After oxidation, there is an increase in the negative charge of the LDL particle, possibly due to the derivatization of positively charged amino groups through the formation of a Schiff base with aldehydes. In addition, oxidative scission of apo B-100 also undergoes during the oxidation of LDL and leads to the fragmentation of apo B-100 in oxidized LDL (53).

2.8.2 Measurement of LDL Oxidation

The evaluation of LDL oxidation *in vivo* is difficult and fraught. The lipoprotein oxidation is likely to occur in the mileu of the artery wall, rather than in the general circulation. In addition, even if some lipoproteins are oxidized in the general circulation, extensively modified lipoproteins are rapidly cleared from the circulation by the scavenger receptors. So the concentration of these modified lipoproteins may not to be easy to detect and may not reflect the extent of oxidation occurring in the arterial wall. In the animal models, samples of arterial tissue can be obtained to examine the amount of oxidative modifications; however limited samples from human (blood, urine, and expired air) are available. Due to the difficulties encountered in obtaining tissue samples in humans, several indirect measurement of LDL oxidation and antioxidant potential *in vitro* must be used. LDL oxidation has been studied *in vitro* using a variety of agents to initiate the reaction such as copper and iron, which can promote rapid lipid peroxidation. In addition, 2,2'-azobis(2-amidiopropane) (AAPH), a water-soluble azo

compound that thermally decomposes has been used in a metal-independent system and lead to the formation of aqueous peroxyl radicals at a constant rate. Various methods to quantify LDL oxidation have been described below.

2.8.2.1 Conjugated Dienes

This method is one of the most widely used for monitoring LDL oxidation. It is easily performed and does not need special extraction step of LDL lipids. Susceptibility of LDL to oxidation *in vitro* and LDL oxidation in samples can be continuously monitored as the kinetics of lipid peroxidation (oxidation of PUFA side chains in LDL particle) by monitoring the formation of conjugated dienes that absorb UV light at the wavelength of 234 nm. In a lag phase, the diene absorption at 234 nm shows only a slight increase; followed by a propagation phase that the absorption at 234 nm rapidly increases. In succession, the 234-nm absorption decreases, then increases again in the decomposition phase because the aldehydes that formed in this phase also absorb in the 210-240 nm region. Currently, this method appears to be the most popular and the best index of LDL oxidizability (53).

2.8.2.2 Thiobarbituric Acid-Reactive Substances

Another widely used index of lipid or lipoprotein peroxidation studies, both in the presence and absence of cells, is the measurement of MDA, a secondary product of lipid peroxidation, by thiobarbituric acid-reactive substances (TBARS) assay with spectrophotometer. The method involves heating the sample with thiobarbituric acid (TBA) under acidic conditions and reading the absorbance of the formed MDA-TBA adduct at the wavelength of 532 nm. TBARS assay is non-specific for only MDA and can be interfered by other compounds in biological sample such as sugars and amino acids that may also form TBA adducts. Precipitation of protein is included in the modification of TBARS assay to minimize interfering protein in the test. The plasma TBARS have shown to be increased in cardiovascular disease patients compared with healthy control in some studies. Although this method is widely used to access lipid peroxidation, it should not be the only measure used because of its lack specificity (53).

2.8.2.3 Relative Electrophoretic Mobility

Native LDL has a negatively charged surface and migrates to the anode in agarose gel electrophoresis under non-denaturing conditions. Oxidation makes LDL more negatively charged, accordingly its electrophoretic mobility increases. The increase of negative charge on Ox-LDL is possibly because of derivatization of lysine residues of apo B-100 by some reactive aldehydes that occured during oxidation. Another possibility is that the reactive oxygen species generate convert histidine and proline residues to negatively charged aspartate or glutamate. The relative electrophoretic mobility (REM) is the ratio of the measurable migration distance of Ox-LDL to native LDL. This method is a very reliable way to quantify LDL oxidation *in vitro* but it lacks the sensitivity of an *in vivo* test. Other modifications by aldehydes will also alter the electrophoretic mobility of LDL (53).

2.8.2.4 Apo B-100 Fragmentation

The degree of oxidation of the LDL protein moiety can be followed by an electrophoresis of apo B-100 fragmentation. The oxidation of LDL causes the scission of apo B-100, leading to apo B-100 fragmentation which can evaluate through an electrophoretic method. Native LDL which has normal apo B-100 will show the band of apo B-100 protein on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) clearly, whereas the band of apo B-100 of Ox-LDL will be completely disappeared or show minimal band. In this electrophoresis method, the area of the band of apo B-100 protein on SDS-PAGE converts inversely to the fragmentation of apo B-100 that occurred.

CHAPTER III

MATERIALS AND METHODS

This project divided into 5 phases as follows:

Phase I: Extraction and identification of γ-OR in samples by reverse-phase HPLC

Phase II: Bioaccessibility and bioavailability of γ-OR

Phase III: Effect of γ-OR on the micellarization and cellular uptake of cholesterol

Phase IV: Effect of γ-OR and ferulic acid on the HMG-CoA reductase activity

Phase V: Antioxidant activities of γ-OR against LDL oxidation

3.1 Phase I: Extraction and Identification of γ-OR in Samples by Reverse-Phase HPLC

3.1.1 Experimental Design

Both chyme and micelle fractions of simulating digested food samples, and cell homogenate as well were extracted and separated for γ-OR by solvent extraction, and then identified for individual components of γ-OR using an analytical reverse-phase HPLC (3).

3.1.2 Materials

Gamma-oryzanol standard, crystalline powder was purchased from Spectrum Lab Products, Inc. (Gardena, CA, USA). Acetone, acetonitrile, dichloromethane, glacial acetic acid, methanol, and petroleum ether purchase from Fisher Scientific (Pittsburgh, PA, USA). Reagents for mobile phase were HPLC grade, except glacial acetic acid that was analytical grade. The mobile phase mixture was filtered with 0.20 μM pore size, hydrophilic nylon membrane (Millipore Corporation, Billerica, MA, USA) before used in HPLC system.

3.1.3 Method

Gamma-oryzanol in a sample was extracted by adding 5 mL of petroleum ether: acetone (2:1) mixture into 1-2 mL of the sample, vortexing at 2500 rpm for 2 minutes in a DVX-2500 multitube vortexer (VWR Scientific, West Chester, PA, USA), and then centrifuging with a Fisher centrific 225 centrifuge (Fisher Scientific, Pittsburgh, PA, USA) at 2000x*g* for 5 minutes at room temperature. Supernatants were collected and the residues were repeatedly extracted for total of 3 times. Pooled extracts were evaporated under nitrogen gas, reconstituted in mobile phase that consists of methanol, acetonitrile, dichloromethane, and glacial acetic acid (50:44:3:3, v/v).

The extracts were injected and analyzed by a reverse-phase high performance liquid chromatography (reverse-phase HPLC) system with a separator (Waters 2695 Separations Module) (Alliance, Waters Corporation, Milford, MA, USA) (Figure 3.1). Components of γ-OR (injection volume of 10 μL) were separated using a Microsorb-MV C18 analytical column (5 μm pore size, 100A°, 4.6 x 250 mm ID) (Varian Analytical Instruments , Inc., Walnut Creek, CA, USA) attached to a pre-column (Waters, 4 μm pore size, 3.9 x 20 mm) (Waters Corporation, Milford, MA, USA) at 25 ºC. Isocratic analyses were performed at a flow rate of 1.4 mL/minute for 55 minutes with a mobile phase that consists of methanol, acetonitrile, dichloromethane, and glacial acetic acid (50:44:3:3, v/v). Gamma-oryzanol components were detected at wavelength 325 nm with a photodiode array UV detector (PAD) (Waters 2996 module, Waters Corporation, Milford, MA, USA), and Empower Pro® version 5.0 for data processing. Components of γ-OR in samples were identified according to data that reported by Xu and Godber (3) and quantified from their peak area using γ-OR standard curve at concentrations of 0.125, 0.25, 0.5, 2, and 4 mg/mL.

Figure 3.1 HPLC –PAD (photographed in the Main Laboratory, Department of Human Nutrition, The Ohio State University, Columbus, OH, USA, 2007)

3.2 Phase II: Bioaccessibility and Bioavailability of γ-OR

3.2.1 Experimental Design

Bioaccessibility or the efficiency of micellarization, and bioavailability or cellular uptake of γ-OR were determined after simulated digestion (Figure 3.2).

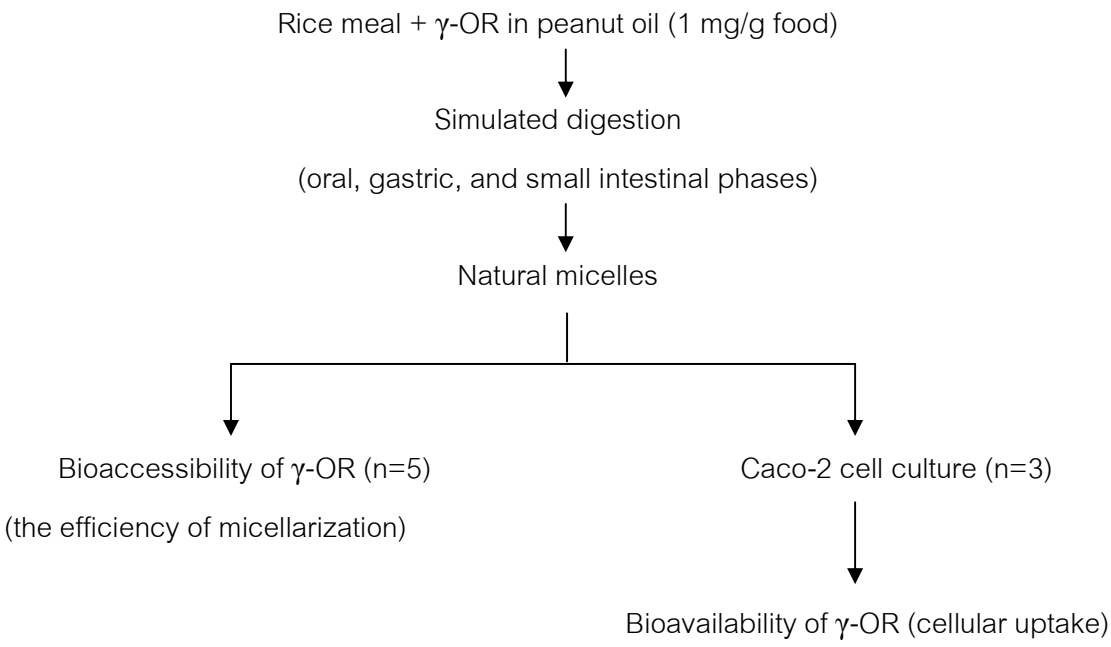


Figure 3.2 Experimental design for bioaccessibility and bioavailability of **γ**-OR

3.2.2 Materials

Gamma-oryzanol standard, reagents for mobile phase used in the HPLC system were purchased as mentioned in section 3.1.2. Glycodeoxycholate (GDC) and taurodeoxycholate (TDC) were purchased from EMD Chemicals, Inc. (San Diego, CA, USA). Supplements and antibiotic for cell culture were purchased from Gibco®, Invitrogen Corporation (Chicago, IL, USA). Other chemicals and medium that used in the simulated digestion and cellular uptake experiments were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Fisher Scientific (Pittsburgh, PA, USA), unless stated otherwise. Filter papers and filtration units with the pore size of 0.22 μm were purchased from Millipore Corporation (Billerica, MA, USA). Materials such as disposable pipettes, disposable syringes, centrifuge tubes, glass vials, T-75 flasks, 6-wells plates, 96-wells plates, and general laboratory materials were purchased from Fisher Scientific (Pittsburgh, PA, USA), ISC Bioexpress® (Kaysville, UT, USA) or Sarstedt (Newton, NC, USA)

3.2.3 Methods

3.2.3.1 Preparation of Food Sample and Vehicle Oil Containing γ-OR

Because of homogeneity of food sample to be digested was essential for the experiment, rice powder from cooked white rice was prepared (Figure 3.3). Jasmine white rice was cooked by adding 2 volumes of distilled water and steamed in rice cooker. Cooked rice was dried in a cooking oven at 105˚C for 5 hours. Then dried cooked rice was blended and sieved with 20-mesh sieve until yielding the fine rice powder. The fine rice powder was kept in a sealed light protected container. Rice meal as food sample for experiment was prepared by adding distilled water into rice powder (3:1, v/w). The food matrice was mixed to be the homogenous rice meal sample. Characteristics of prepared rice meal are shown in Figure 3.4.

Due to the similar profiles of fatty acids in peanut oil and RBO, peanut oil with absence of γ-OR (Hollywood® brand, The Hain Celes Tial Group, Inc., Melville, NY,

USA) was used as vehicle oil in this study. Based on the labeled nutrition fact, peanut oil used as the vehicle oil in this study was composed of SFA 2 g, *trans*-fat 0 g, PUFA 5 g, and MUFA 7 g per 15mL. Gamma-oryzanol standard powder was dissolved in peanut oil as stock solution at the concentration of 25 mg/mL peanut oil. The solution was warmed at 50˚C to increase solubility of γ-OR in peanut oil in a water bath (Thermo-Precision Module, Thermo Electron Corporation, Marietta, OH, USA) until γ-OR was completely dissolved. Clear high γ–OR peanut oil was observed. The prepared high γ-OR oil was kept at -20˚C until used. Solubility and clearness of the peanut oil containing γ-OR were experienced after thawed at room temperature before using in the experiments.

A. *Drying cooked jasmine rice in the oven*

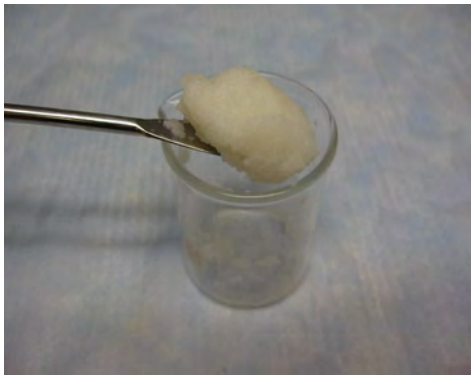
B. *Dried cooked rice*

C. *Dried cooked rice before blending*

D. *Blending dried cooked rice*

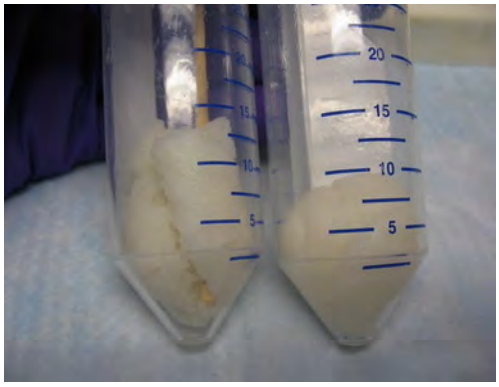
E. *Sieved rice powder*

Figure 3.3 Preparation of rice powder samples from the cooked jasmine rice



A. *Prepared rice meal from rice powder*

B. *Prepared oil solutions for experiments*



C. *Mixture of rice meal and a prepared oil solution before simulated digestion*

Figure 3.4 Characteristics of prepared rice meal samples and oil solutions

3.2.3.2 Simulated Digestion

Complete simulated digestion including the oral, gastric, and small intestinal phases of digestion was conducted according to procedure described elsewhere with minimum modifications (Figure 3.5) (40, 41, 55).

Oral phase: Base solution of synthetic saliva containing 0.012 M KCl, 0.002 M KSCN, 0.007 M NaH_2PO_4, 0.004 M Na_2SO_4, 0.005 M NaCl, 0.002 M NaOH, pH 6.5 was prepared. Two grams of prepared rice meal was weighed into each 50 mL screw-cap polypropylene tube. Eighty microliters of prepared high γ-OR peanut oil or regular peanut oil were added into the homogenized rice meal and mixed well. Final concentration of γ-OR in the tested rice meal was 1 mg/g food. Oral phase of digestion began by addition with 7 mL of synthetic saliva (0.015 mg/mL uric acid, and 0.05 mg/mL

mucin in base solution plus 522 units of α-amylase per 1 g digested food). Sample tubes were blanketed with nitrogen, sealed, mixed, and placed horizontally in shaking water bath (Thermo-Precision Module, Thermo Electron, Marietta, OH, USA) with speed of 60 rpm at 37˚C for 15 minutes. After the ending of incubation period of the oral phase, the sample tubes were removed and placed on ice immediately and then continued the simulated gastric digestion.

Gastric phase: Digested samples from oral phase were acidified to pH 2.0 ± 0.5 with 1M HCl. Two milliliters of porcine pepsin (20 mg/mL in 100 mM HCl) were added and increased volume of reaction to 20 mL with solution A (120 mM NaCl, 6 mM $CaCl_2$, and 5 mM KCl) in each tube. Final concentration of pepsin was 2 mg/mL. Sample tubes were blanketed with nitrogen gas, sealed, mixed, and placed horizontally in shaking water bath (60 rpm) at 37˚C for 1 hour. After 1 hour, the reaction tubes were placed immediately on ice and continued simulated small intestinal digestion.

Small intestinal phase: The pH of digested samples from gastric phase were immediately increased to 6.0 ± 0.2 with 1M $NaHCO_3$, followed by the addition of a mixture of bile salts (3 mL of mixture containing 6.3 mg/mL glycodeoxycholate (GDC), 3.9 mg/mL taurodeoxycholate (TDC) and 6.7 mg/mL taurocholate (TC) in 100 mM $NaHCO_3$), and a mixture of porcine pancreatin and pancreatic lipase (2 mL of mixture containing 10 mg/mL porcine pancreatin, and 5 mg/mL porcine pancreatic lipase in 100 mM $NaHCO_3$). The pH of each sample tube was increased to 6.5 ± 0.5 with 1M NaOH if needed, and the final volume of reaction in the sample tubes were increased to 50 mL with solution A. Finally, total 50 mL of reaction mixture contained 0.8 mM GDC, 0.45 mM TDC, 0.75 mM TC, 0.4 mg/mL pancreatin, and 0.2 mg/mL lipase. Sample tubes were blanketed with nitrogen gas, sealed, and incubated in a shaking water bath (60 rpm) at 37 ºC for 2 hours. After 2 hours, the reaction tubes were placed immediately on ice and ready for preparation of micelle fraction.

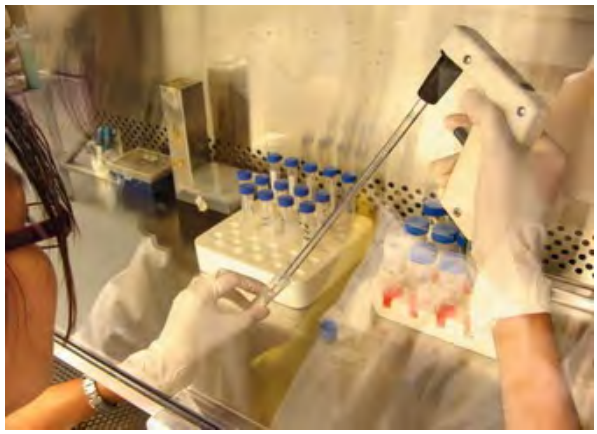
Preparation of micelle fraction: After completion of the simulated digestion, the product was referred to as the "chyme." The chyme were centrifuged at 5000x*g* for 45 minutes at 4°C to separate the aqueous fraction containing mixed micelles from residual

solids and oil droplets. The aqueous fraction or micelle fration was collected and passed through cellulose acetate filter (0.22-μm pore size) to remove microcrystalline γ-OR aggregates and microbial contaminants. The chyme from simulated digestion and the filtered aqueous or micelle fraction were sealed under a stream of nitrogen and stored at –80°C until analysis within 1 week. The aqueous fraction used as a source of micellarized γ-OR was diluted with medium and treated differentiated Caco-2 cells for bioavailability and cellular uptake experiments (section 3.2.3.4). The amounts of γ-OR in chyme and micelle fractions were analyzed by HPLC. Then efficiencies of micellarization were calculated.

A. *Adding synthetic saliva into rice meal*

B. *Incubating samples during simulated digestion*

C. *Filtrating the micelle fraction*



D. *Preparing micelles-medium mixture for the Caco-2 cellular uptake experiment*

Figure 3.5 Simulated digestion

3.2.3.3 Cell Culture Preparation

Stock cultures of Caco-2 cells (ATCC HTB-37, Rockville, MD, USA) were maintained in T75 flasks (vented caps) containing high-glucose Dulbecco's modified Eagle's medium (DMEM; Sigma D-7777) supplemented with 15% heat-inactivated fetal bovine serum (FBS), 4 mM L-glutamine (Gibco® 25030-081), 1% non-essential amino acids (NEAA; Gibco® 11140-050), 100 U/mL penicillin-streptomycin (Gibco® 15290-018), 0.5 μg/mL fungizone (Gibco® 15290-018), 44 mM sodium bicarbonate, and 15 mM HEPES (Sigma H-3375) in a humidified atmosphere of 95% air and 5% CO_2 at 37˚C (Thermo Scientific Forma® 3110 Series II Water Jacketed CO_2 Incubator with HEPA filter, Thermo Fisher Scientific, Inc.).

Cells were split to establish new stock culture when the cell monolayer reached 70-80% confluent. The growth medium was not changed from seeding to split. For experiments, stock cultures were used to seed at passages 20 – 35 in the medium as described above with medium changed every other day. After monolayers were confluent, FBS content of medium was decreased to 7.5%. Cells were used for uptake experiment at 11-14 days after reaching 100% confluent. Medium were changed the day before experiments. Maintenance of Caco-2 cell line for experiments was done as previously described (40, 41).

3.2.3.4 Cellular Uptake of Micellarized γ-OR

Cultures of Caco-2 cell line at passages 20 – 35 were seeded in T75 flasks at 4×10^3 cells/cm^2 in the medium as described above for using in the experiment. Cell culture experiment was done in a Biological safety cabinet (Thermo Scientific Forma® 1184 Class II A/B3 Biological Safety Cabinet, Thermo Fisher Scientific, Inc.). Prepared micelle fraction was diluted 1:4 with DMEM containing 4 mM L-glutamine and 1% non-essential amino acids, but no FBS or antibiotics. The differentiated monolayers of Caco-2 cells were washed once with DMEM (no supplements) at 37˚C and then DMEM was replaced with test medium. During an incubation period in conventional condition, cell cultures were observed microscopically to ensure monolayers intacted (Figure 3.6).

Schematic diagram of a Caco-2 intestinal cell absorption model was shown in Figure 3.7.

After incubation for 4 hours, cell culture plates were placed on ice. The test medium was removed and monolayers were washed once with cold Dulbecco's phosphate buffered saline (PBS; Mediatech, Inc., Herndon, VA, USA) containing bovine serum albumin (2 g/L) to reduce non-specific adsorption of micelles to cell surface. The monolayers were washed two additional times with cold standard PBS. Monolayers were harvested in small volume of PBS and cells were pelleted by centrifugation at 1000x*g*, 4˚C, for 5 minutes (Jouan GR-412, Jouan Inc., Winchester, VA, USA). The supernatant was discarded; cell pellets were blanketed with nitrogen gas. The sealed tubes were stored at -20˚C for a maximum of 7 days (40, 41).

On the day of analysis, cell pellets were thawed and sonicated briefly by a probe sonicator (Vibra-Cell™, Sonics and Materials, Inc., Newtown, CT, USA) in 1 mL ice-cold PBS. An aliquot of each cell homogenate was taken to determine the protein content by the bicinchoninic acid (BCA) protein assay (Pierce, Thermo Fisher Scientific, Inc., Rockford, IL, USA), using bovine serum albumin as a standard (56). Gamma-oryzanol in each cell homogenate was extracted and analyzed by HPLC as described above. The efficiencies of γ-OR incorporating into micelles (bioaccessibility) and cellular uptake of γ-OR (bioavailability) were calculated using the amount of γ-OR in the test medium and in the treated Caco-2 cells standardized by protein content of each cell homogenate.

3.3 Phase III: Effect of γ-OR on the Micellarization and Cellular Uptake of Cholesterol

3.3.1 Experimental Design

This study determined the effect of γ-OR on the cholesterol micellarization in both natural micelles from simulated digestion and synthetic micelles by monitoring added ^{14}C-cholesterol trace in the test samples. Uptake of cholesterol was further evaluated by determination of ^{14}C-cholesterol in harvested cells at indicated time (Figure 3.8).

A. *Washing the monolayers of differentiated Caco-2 cells and replacing the old medium with tested micelles-medium mixture*

B. *Hourly microscopical observation of cell cultures during incubation period to ensure the intact of the monolayers*

Figure 3.6 Caco-2 cellular uptake of micellarized γ-OR

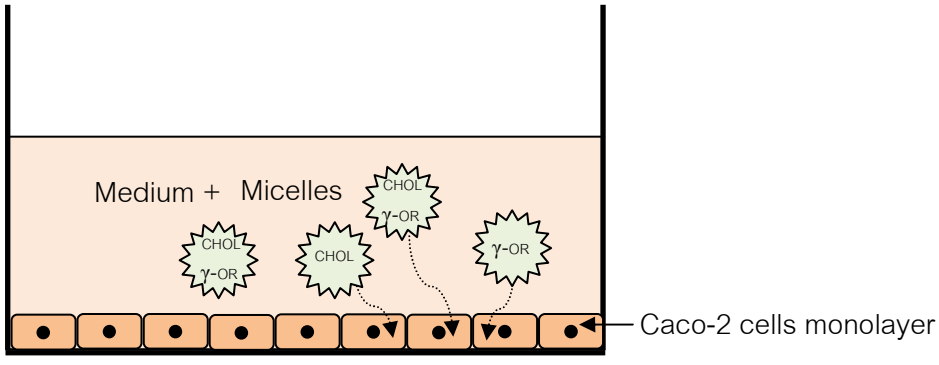


Figure 3.7 Schematic diagram of a Caco-2 intestinal cell absorption model

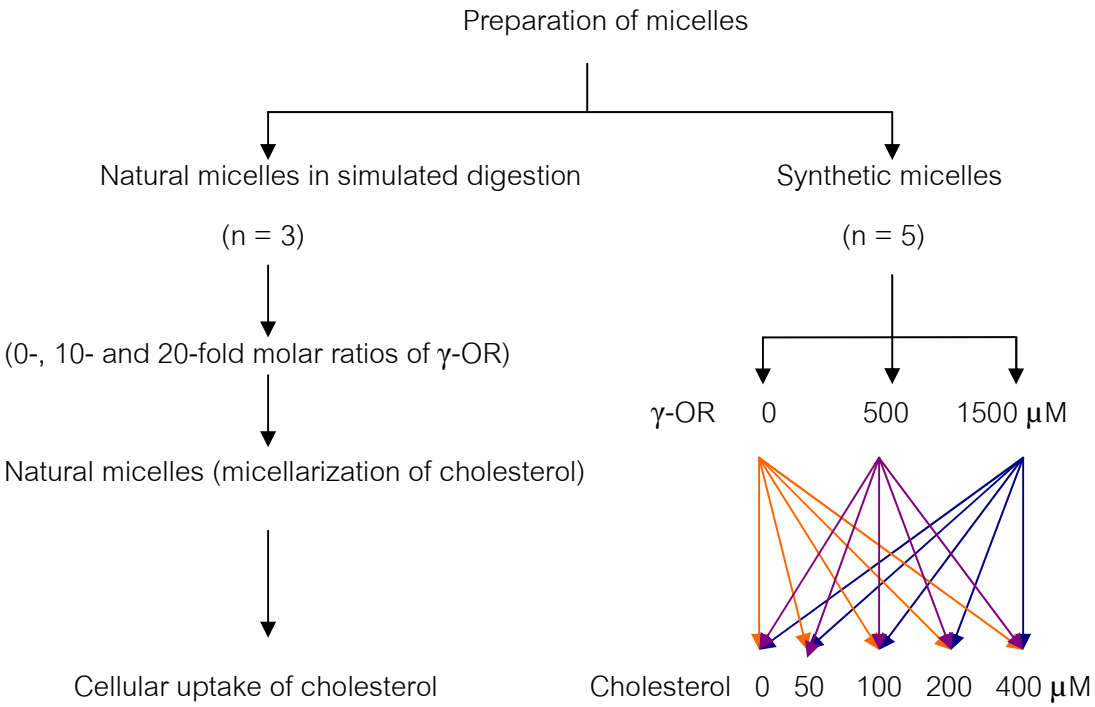


Figure 3.8 Experimental design for the effect of γ-OR on micellarization and cellular uptake of cholesterol

3.3.2 Materials

^{14}C-cholesterol in ethanol form was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA, USA). Gamma-oryzanol standard (M.W. 602.98), crystalline powder was purchased from Spectrum Lab Products, Inc. (Gardena, CA, USA). Glycodeoxycholate (GDC) and taurodeoxycholate (TDC) were purchased from EMD Chemicals, Inc. (San Diego, CA, USA). L-α-Phosphatidylcholine from egg 99% (PC) and lyso-phosphatidylcholine (Lyso-PC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Supplements and antibiotic for cell culture were purchased from Gibco®, Invitrogen Corporation (Chicago, IL, USA). Other chemicals and medium that used in the simulated digestion and cellular uptake experiments were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Fisher Scientific (Pittsburgh, PA, USA), unless stated otherwise.

Materials such as disposable pipettes, disposable syringes, centrifuge tubes, glass vials, T-75 flasks, 6-wells plates, 96-wells plates, and general laboratory materials

were purchased from Fisher Scientific (Pittsburgh, PA, USA), ISC Bioexpress® (Kaysville, UT, USA) or Sarstedt (Newton, NC, USA). Filter papers and filtration units were purchased from Millipore Corporation (Billerica, MA, USA).

3.3.3 Methods

3.3.3.1 Stock Solution Preparation

^{14}C-cholesterol (specific activity 53 mCi/mmol ^{14}C-cholesterol; 0.04 mCi/mL in ethanol) was prepared at the indicated radioactivity for the experiments. Stock solutions of other chemicals such as monoolein (MO), phosphatidylcholine (PC), lyso-phosphatidylcholine (Lyso-PC), α-tocopherol (TC), γ-OR, and normal cholesterol (M.W. 386.67) were prepared in chloroform.

3.3.3.2 Synthetic Micelles Preparation with Radioisotope ^{14}C-Cholesterol

Preparation of synthetic micelles was based on several *in vitro* studies (40, 41, 57). Synthetic micelle solution containing final concentrations of 500 μM MO, 100 μM PC, 300 μM Lyso-PC, 10 μM α-tocopherol, γ-OR at concentrations of 0, 500, and 1500 μM), normal cholesterol at concentrations of 0, 50, 100, 200, and 400 μM, and 0.01 μCi of ^{14}C-cholesterol were prepared by combination of the stock solutions in an individual 15 mL flat bottom vial. Solvents were evaporated under nitrogen stream at room temperature. Calculated volume of basal medium containing 1% NEAA, 1% L-glutamine, 0.8 mM GDC, 0.46 mM TDC, 0.75 mM TC, and 0.6 mM sodium oleate (OA) were added to each individual vial. Then, the mixtures were sonicated in an ultrasonic bath (Bransonic® 12, Branson Ultrasonic Corp., Danbury, CT, USA) at room temperature for 30 minutes. The solution were centrifuged at 10000x*g*, 4°C, for 15 minutes (Eppendorf centrifuge 5417C, Eppendorf North America, Hauppauge, NY, USA) to remove insoluble γ-OR and cholesterol aggregates. The supernatant was referred as micelle fraction. The amounts of ^{14}C-cholesterol in micelle fraction and in pre-centrifuged solution were determined after adding the scintillation fluid (ScintiSafe Econo 1 Cocktail, Fisher Scientific), using a liquid scintillation counter (LS 3801, Beckman Coulter, Inc., Fullerton, CA, USA). The efficiencies of cholesterol incorporated into synthetic micelles were

calculated from ^{14}C-cholesterol values in micelle fraction and in pre-centrifuged solution, assuming that ^{14}C-cholesterol traces represented total cholesterol in the system.

3.3.3.3 Preparation of Peanut Oil Containing ^{14}C-Cholesterol

Prepared stock ^{14}C-cholesterol solution (20 μCi/mL) and an aliquot of normal cholesterol standard in ethanol (0.5 mg/mL) were mixed and dried under nitrogen. The film in the glass tube was dissolved with peanut oil by placing in water bath at 50˚C until it was completely dissolved. Peanut oil containing γ-OR (7.84 mg/mL and 15.68 mg/mL) and one containing cholesterol standard (0.5 mg/mL) were prepared as described in the section 3.2.3.1. All prepared oil solutions were stored at -20˚C until used.

3.3.3.4 Simulated Digestion and Cellular Uptake Experiments with ^{14}C-Cholesterol

Simulated digestion and cellular uptake experiments were conducted following the method described in section 3.2.3.2. Two grams of rice meal mixed with peanut oil containing 0-(control), 10- and 20-fold molar ratio of γ-OR to cholesterol with ^{14}C-cholesterol traces (1 μCi) were simulating digested including the oral, gastric, and small intestinal phases. Total volume of peanut oil that used in total 50 mL of reaction mixture were not exceed 100 μL (50 μL of peanut oil containing normal cholesterol plus ^{14}C-cholesterol traces and 50 μL of peanut oil containing γ-OR or regular peanut oil as control). Final concentration of normal cholesterol was 1.3 μM with final concentrations of γ-OR at 0, 13.0, and 26.0 μM in reaction mixture. Radioisotope awareness were concerned to protect environment not be contaminated with radioisotope. Peanut oil containing normal cholesterol was used in a reference tube with the absence of ^{14}C-cholesterol to adjust pH during simulated digestion so the used pH meter (Corning® 445, Corning, Inc., Corning, NY, USA) was not contaminated by radioisotope. After completion of simulated digestion, micelle fraction was obtained as described in section 3.2.3.2.

For cellular uptake experiments, stock Caco-2 cells maintenance and Caco-2 cells monolayers preparation were conducted as described in section 3.2.3.3 and

3.2.3.4. Cultures of Caco-2 cell line at passages 20 – 35 were seeded in 6-well plates at 2.6×10^4 cells/cm^2 in the medium as described above. Schematic diagram of Caco-2 intestinal cell absorption model in this study is shown in Figure 3.7. Differentiated Caco-2 cells were treated with test DMEM containing prepared micelle fraction (25%, v/v) for 4 hours. The micelles-treated Caco-2 cells were harvested after indicated time.

The contents of ^{14}C-cholesterol in chyme, micelle fraction and in each cell homogenate were determined after the addition of scintillation fluid, using a liquid scintillation counter. Protein content of each cell homogenate was also determined using the BCA protein assay (Pierce, Thermo Fisher Scientific, Inc., Rockford, IL, USA). The efficiency of cholesterol incorporating into micelles and cellular uptake of cholesterol were calculated. Cellular uptake of cholesterol was standardized by protein content of each cell homogenate. All radioactive materials and waste were handling and discarded according to the radiation safety standards for The Ohio State University.

3.4 Phase IV: Effect of γ-OR and Ferulic Acid on the HMG-CoA Reductase Activity

3.4.1 Experimental Design

The inhibitory effects of γ-OR, and ferulic acid on HMG-CoA reductase activity *in vitro* were investigated. Crude HMG-CoA reductase enzyme was prepared from microsomes of rat livers (Figure 3.9).

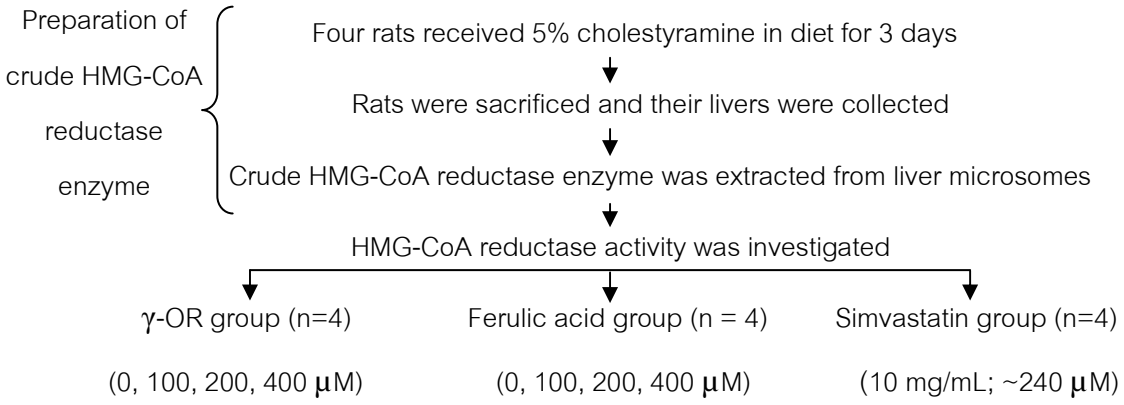


Figure 3.9 Experimental design for the effects of γ-OR and ferulic acid on the HMG-CoA reductase activity

3.4.2 Materials

Gamma-oryzanol standard powder was purchased from Spectrum Lab Products, Inc. (Gardena, CA, USA). Bestatin® 10 containing 10 mg of simvastatin/ tablet (Berlin Pharmaceutical Industry Co., Ltd., Bangkok, Thailand) was used as simvastatin standard. Other chemicals, standards, and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless stated otherwise.

3.4.3 Methods

3.4.3.1 Preparation of γ-OR and Ferulic Acid Stock Solution

Chloroform and dimethyl sulfoxide (DMSO) were used to solubilize γ-OR and ferulic acid, respectively. Simvastatin was solubilized in DMSO and centrifuged 3000 rpm for 10 minutes in a microultracentrifuge (Himac CS100, Hitachi, Ltd., Tokyo, Japan) and supernatant was used in the experiment as a negative control. A pilot study was performed to ensure that prepared stock solutions at indicated concentrations did not cause any precipitation in the assay reaction mixture (see below).

3.4.3.2 Partial Purification of HMG-CoA Reductase

HMG-CoA reductase enzyme was partially purified according to the method described by Kim *et al.* with some modification (58). Four male Wistar rats (180-200 g body weight) were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Thailand. Animals were housed maximum 2 rats per cage in a room with natural light cycle from 06:00 to 18:00. Food and water were available for them *ad libitum*.

Rats were fed powder chow diet containing 5% cholestyramine for three days before sacrificed. They were killed at midnight as a result of the maximum activity HMG-CoA reductase. Each rat liver was finely minced and homogenized manually at 4°C in 25 mL of buffer A (100 mM sucrose, 50 mM KCl, 40 mM potassium phosphate, and 30 mM sodium EDTA, pH 7.2) with a glass-glass homogenizer (Wheaton, Millville, NJ, USA), and the microsomes were prepared. Approximately 3 mL aliquots of microsome

suspension were frozen at a rate of 6-8°C per minute and stored at -20°C. For optimal solubilization of the reductase enzyme, the frozen microsome were thawed at 37°C before adding an equal volume of 50% glycerol in buffer B (buffer A plus 10 mM *dl*-dithiothreitol) preheated to 37°C. The suspension were re-homogenized manually with 10 downward passes of a glass-glass homogenizer and then be incubated at 37°C for 1 hour. The incubated suspension were diluted three-fold with buffer B at 37°C, rehomogenized manually with another 10 downward passes of the glass-glass homogenizer, and centrifuged at 100000x*g* with a microultracentrifuge (Himac CS100, Hitachi, Ltd., Tokyo, Japan) for 60 minutes at 25°C. The supernatant containing solubilized HMG-CoA reductase were collected and used for the enzyme inhibitory activity assay as the crude enzyme.

3.4.3.3 Activity Assay of HMG-CoA Reductase

The inhibitory activity assay of HMG-CoA reductase was performed according to the method that previously described by Kim *et al.* (58). Its activities were determined at 37°C in a total volume of 0.5 mL using a spectrophotometer (Hitachi U-2001 Spectrophotometer, Hitachi, Ltd., Tokyo, Japan). The activity assay reaction mixture contained 200 mM KCl, 160 mM potassium phosphate, 4 mM sodium EDTA, and 1 mM *dl*-dithiothreitol, pH 6.8, 0.2 mM NADPH, 0.1 mM HMG-CoA, and γ-OR (0, 100, 200, 400 μM), or ferulic acid (0, 100, 200, 400 μM), or simvastatin (~240 μM). The activities of HMG-CoA reductase were determined by following the oxidation rate of NADPH which could be monitored by decrease of absorbance at wavelength 340 nm. The absorbance of reaction mixtures with presence of NADPH was initially determined in the absence of HMG-CoA. This blank value was subtracted from those that obtained with both NADPH and HMG-CoA. The percentage of inhibitory HMG-CoA reductase activity was calculated by a following formula:

$$\% \text{ inhibition of HMG-CoA reductase activity} = (Abs_C - Abs_I) \times 100 / Abs_C$$

Where Abs_C was an absorbance measured from control reaction (reagent blank; no γ-OR, no ferulic acid, no simvastatin) and Abs_I was an absorbance measured from the reaction with any indicated concentrations of γ-OR, ferulic acid, or simvastatin.

3.5 Phase V: Antioxidant Activities of γ-OR against LDL Oxidation

3.5.1 Experimental Design

Antioxidant activities of γ-OR against LDL oxidation were compared with those of ferulic acid and α-tocopherol at various concentrations. The individual LDL fraction separated from blood of healthy male volunteers was used in the susceptibility tests to oxidation in this study (Figure 3.10).

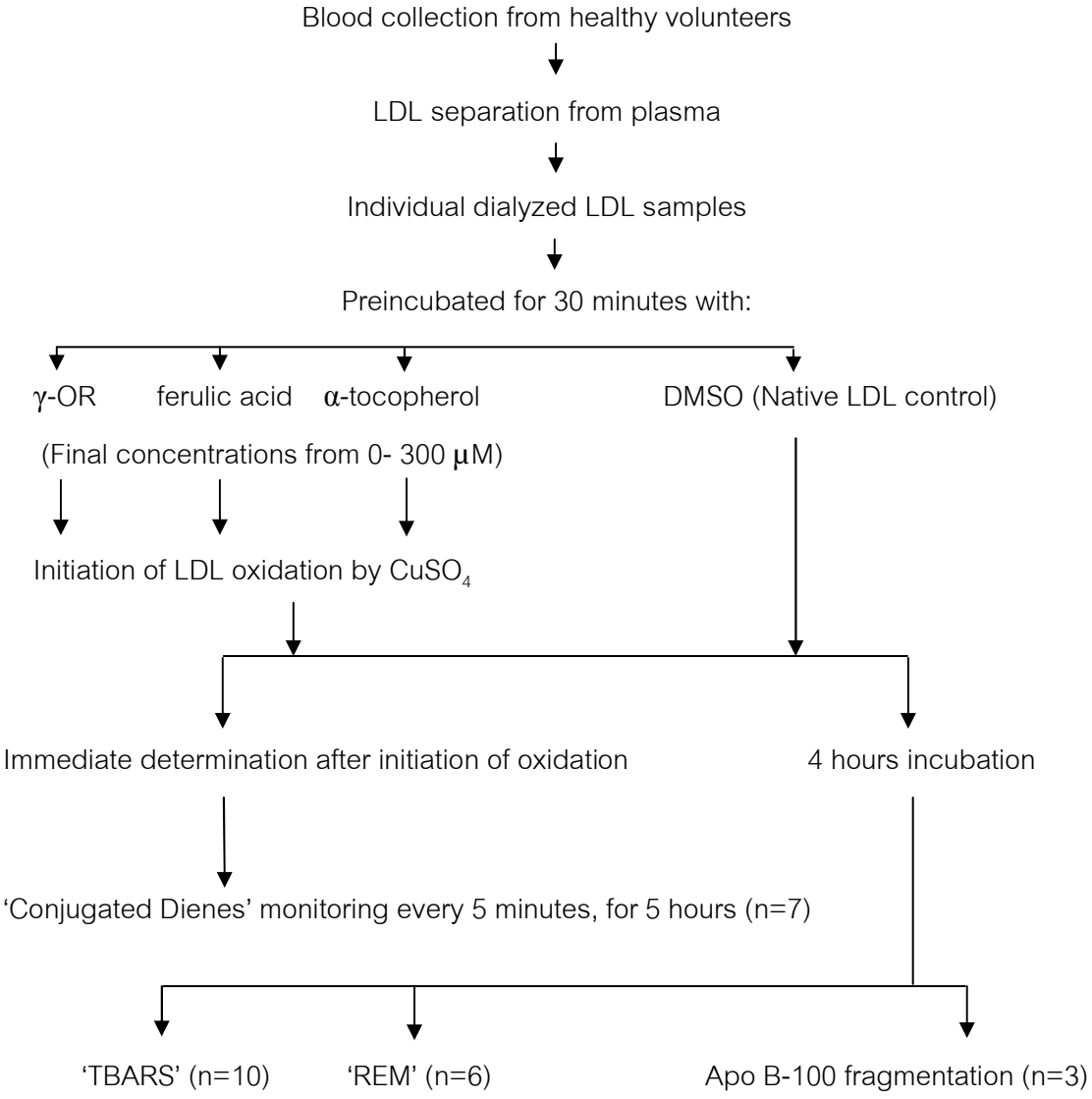


Figure 3.10 Experimental design for antioxidant activities of γ-OR against LDL oxidation

3.5.2 Materials

Gamma-oryzanol standard was purchased from Spectrum Lab Products, Inc. (Gardena, CA, USA). Reagents for electrophoresis experiments were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Other chemicals, standards, and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless stated otherwise.

3.5.3 Methods

3.5.3.1 Blood Collection

Fifteen healthy volunteers age between 19 - 28 years old were recruited by advertisements at Faculty of Allied Health Sciences, Chulalongkorn University. All information about the purpose, the risks of this study, the privacy policy, and the right of participants were explained to the subjects. Written informed consents were obtained from subjects before participation. The study was approved and conducted according to the requirements of the Ethical Review Committee for Research Involving Human Research Subjects, Health Science Group, Chulalongkorn University, Thailand.

The inclusion criteria for participation were:

1. Fasting plasma total cholesterol concentration below 200 mg/dL, and plasma triacylglycerol concentration below 150 mg/dL
2. Fasting blood glucose less than or equal 100 mg/dL
3. No history of following diseases; diabetes mellitus, hyperlipidemia, cardiovascular diseases, liver function disorders, hyperthyroidism, hypothyroidism, cancer
4. Not use lipid-lowering drug and any medication that might affect lipid metabolism
5. Normal blood pressure (between 100/60 and 140/90 mm-Hg)
6. Non smoker

7. Not consume any food supplements or antioxidants products for 1 month before enrollment and during participation
8. Have enough night sleep before the date of blood collection
9. Agree to join the study and give informed consent

Fifteen milliliters of 12 hours-fasting blood were collected in EDTA containing tubes (1 mg/mL blood placed in an ice-box, and kept at 4°C throughout its preparation. Blood samples collected from each subject were prepared separately. Plasma separated by centrifugation at 3000x*g*, 4°C for 10 minutes. Plasma samples were kept in the tubes covered with aluminum foil and stored at -80°C under nitrogen seal until use but not more than 30 days.

3.5.3.2 LDL Preparation

The LDL fraction, corresponding to a density of 1.019-1.063 g/mL was isolated from plasma by sequential ultracentrifugation in KBr solution following a series of sample density adjustments, centrifugation, and infranatant/supernatant separation (59). Briefly, the density of plasma was adjusted to be 1.019 g/mL by addition of indicated amount of solid KBr that calculated by the equation 1; assuming that the density of plasma = 1.006 g/mL).

$$KBr\ (g) = V\ (D_f - D_i)/\ 0.7259 \text{ ---------- equation 1}$$

V, plasma volume (mL); D_f, final density (1.019 g/mL); D_i, initial density (1.006 g/mL)

Final weights of each microultracentrifuge tube that contained samples were adjusted by addition of 1.019 g/mL KBr solution (about 1 mL for each tube). Then, 1.019 g/mL density-adjusted plasma samples were centrifuged at 100000 rpm, 4°C, in a S100AT5 angle rotor using Himac CS100 microultracentrifuge (Hitachi, Ltd., Tokyo, Japan), for 3 hours. The upper fraction of each tube, containing triacylglycerol-rich lipoproteins, was removed. The lower fraction (infranatant) that contained LDL, HDL and plasma proteins was collected for the next step.

Consequently, the density of infranatant from previous separation was adjusted to be 1.063 g/mL by addition of indicated amount of solid KBr that calculated by the equation 2.

$$KBr\ (g) = V\ (D_f - D_i)/\ 0.7057 \text{ ---------- equation 2}$$

V, initial volume of the infranatant (mL); D_f, final density (1.063 g/mL); D_i, initial density (1.019 g/mL)

Final weights of each microultracentrifuge tube that contained samples were adjusted by addition of 1.063g/mL KBr solution (about 1 mL for each tube). Then, 1.063g/mL density-adjusted samples were centrifuged at 100000 rpm, 4˚C, in a S100AT5 angle rotor using Himac CS100 microultracentrifuge (Hitachi, Japan) for 4 hours. The upper fraction from this step, containing LDL lipoproteins, was collected. The lower fraction (infranatant) that contained HDL and plasma proteins was discarded.

Plasma samples were kept at 4ºC throughout LDL fraction preparation to diminish lipoprotein modification. The LDL samples were stored under nitrogen and sealed in the aluminum foil covered tubes at 4ºC until used for LDL dialysis within 1 week after LDL isolation. Dialyzed LDL samples were kept under nitrogen and sealed in the aluminum foil covered tubes at 4ºC and were used for the experiment within 1 week after LDL dialysis. Protein content in dialyzed LDL samples was measured by Bio-Rad protein assay (Bio-Rad Laboratories, Inc., CA, USA), according to the method of Bradford (60). Bovine serum albumin was used as the standard material. Lipoprotein concentrations in dialyzed LDL samples were expressed in terms of protein content.

3.5.3.3 LDL Dialysis

Before the susceptibility of LDL to *in vitro* oxidation experiments, LDL samples were dialyzed by passing through Sephadex™ PD-10 columns (Amersham Biosciences Inc., Piscataway, NJ, USA) (61) that equilibrated with cold phosphate buffered saline (PBS) (containing 0.01 M phosphate, 0.0027 M KCl, and 0.138 M NaCl; pH 7.4), for two times to remove EDTA and other interfering compounds. EDTA-free dialyzed LDL

samples were diluted in PBS as indicated concentration in the *in vitro* oxidation experiments. Tested compound; α-tocopherol, γ-OR, and ferulic acid were dissolved in DMSO. In reaction mixture, dialyzed LDL samples were pre-incubated for 30 minutes with final concentrations of a range from 0-300 μM of α-tocopherol, γ-OR, ferulic acid solutions in DMSO at 37˚C before initiation of LDL oxidation.

3.5.3.4 Conjugated Dienes Determination of *in vitro* Oxidized LDL

Four hundred and ninety microliters of dialyzed LDL samples (final concentration at 50 μg LDL protein/ mL) were pre-incubated with 5 μL of α-tocopherol, γ-OR, ferulic acid solutions in DMSO (0, 1, 5, 10, 50 μM final concentrations) for 30 minutes at 37˚C. The *in vitro* oxidation of dialyzed LDL samples were initiated by adding 5 μL of freshly prepared aqueous $CuSO_4$ solution (10 μM final concentration) into quartz cuvettes contained LDL samples suspension at 37˚C, and mixed gently with covered parafilm. Distilled, de-ionized water (DDI) was used instead of $CuSO_4$ solution in the LDL samples suspension for negative control (native LDL). Each experiment set of each LDL sample was performed in duplicate.

Since LDL oxidation initiated, kinetic of LDL oxidation were observed immediately by monitoring the increased formation of conjugated dienes at 234 nm using a UV-Visible spectrophotometer (Evolution 600 model, coupled with a rotary 7-cell changer, and TPS 1500W Peltier Cryobath System-Temperature controller, with VISIONpro™ software for data processing, Thermo Fisher Scientific, Inc., England) at 37˚C using the method of Esterbauer *et al*. (62). The initial absorbance of individual sample was taken at 234 nm as the baseline absorbance at T_0 and the change in absorbance were recorded every 5 minutes for 5 hours. The recorded absorbance at each time point was subtracted by the baseline absorbance at T_0 before plotting the graph (minutes and subtracted absorbance on x-axis, and y-axis, respectively). The absorbance graph of samples at 234 nm was divided into three phases; a lag phase, a propagation phase, and a decomposition phase. The oxidation lag phase (lag time) was defined as the intercept of the tangent of the slope of the absorbance curve in propagation phase with the baseline, and data was expressed in minutes. The

propagation rate of conjugated dienes formation can be calculated from the slope of the absorbance curve during the propagation phase, expressed as nmol conjugated dienes· min^{-1}· g^{-1} of LDL protein. The concentration of conjugated dienes can be calculated using the molar extinction coefficient of conjugated dienes at 234 nm (ε_{234} = 29500 $L \cdot mol^{-1} \cdot cm^{-1}$)(63). Decomposition time (minutes) defined as the time when the maximal conjugated dienes produced and then began to decline during the decomposition phase. The conjugated dienes production during LDL oxidaiton (the maximal amount of conjugated dienes formed) can be calculated using the molar extinction coefficient of conjugated dienes, and expressed as nmol· g^{-1} of LDL protein.

3.5.3.5 TBARS Determination of *in vitro* Oxidized LDL

Ninety microliters of dialyzed LDL samples (final concentration at 300-500 μg LDL protein/ mL) were pre-incubated with 5 μL of α-tocopherol, γ-OR, ferulic acid solutions in DMSO (0, 1, 10, 100, 200, 300 μM final concentrations) for 30 minutes at 37˚C. The *in vitro* oxidation of dialyzed LDL samples were initiated by adding 5 μL of freshly prepared aqueous $CuSO_4$ solution (30 μM final concentration) into LDL samples and incubated at 60˚C for 4 hours. Individual LDL sample that incubated with DMSO and DDI instead of tested compounds and $CuSO_4$ solution was used as their own native LDL samples. After incubation of 4 hours, TBARS contents of native, and Ox-LDL in treated LDL samples were assessed by TBARS assay according to the method that described by Scoccia *et al.* (64) with some modification. Incubated LDL samples and MDA standard (Malondialdehyde tetrabutylammonium salt: Sigma-Aldrich, Cat. No. 63287) were added with 250 μL of 20% Trichloroacetic acid (TCA) and 250 μL of 0.78% aqueous solution of thiobarbituric acid (TBA) and then heated the reaction tubes in a water bath with covered glass beads. After heating at 95˚C for 45 minutes, the reaction tubes were cooled to room temperature in an ice bath. Then, samples were centrifuged at 4000 rpm for 5 minutes using Himac CF7D2 centrifuge (Hitachi, Ltd., Tokyo, Japan). Supernatnatant fractions were collected and transferred to 96-wells micro-titers plates. The red pigment of TBARS contents in the supernatant fractions was determined at 532 nm with Microtiter-plates reader (Biotek® Power Wave 340 Module, BioTek Instruments,

Inc., Winooski, VT, USA) with Gen5™ program version 1.04.5 (BioTek Instruments, Inc., Winooski, VT, USA) for data processing.

A calibration curve was prepared every time of each experiment set, using 6 concentrations (0, 1.25, 2.5, 5, 10, 100 nmol/mL) of MDA standard in PBS. Each experiment set of each LDL sample was performed in triplicate. Results were expressed as nmol MDA/ mg LDL protein. A recovery test was also performed.

3.5.3.6 Determination of Relative Electrophoretic Mobility of LDL

The electrophoretic mobility of native or oxidized LDL was detected by agarose gel electrophoresis according to the method described by Chu *et al*. (65) with some modification. Ninety microliters of dialyzed LDL samples (final concentration at 300 μg LDL protein/ mL) were pre-incubated in microcentrifuge tubes with 5 μL of γ-OR (1, 10, 100, 200, 300 μM final concentrations), α-tocopherol or ferulic acid solutions in DMSO (1, 10, 100 μM final concentrations) for 30 minutes at 37˚C. The *in vitro* oxidation of dialyzed LDL samples were initiated by adding 5 μL of freshly prepared aqueous $CuSO_4$ solution (30 μM final concentration) into LDL samples and then incubated at 37˚C for 4 hours in a VorTemp56™ shaker incubator (Labnet International, Inc., Woodbridge, NJ, USA). Individual LDL sample that incubated with DMSO and DDI instead of tested compounds and $CuSO_4$ solution was used as their own native LDL samples.

After 4 hours of incubation, 40 μL of samples were loaded onto each well of agarose gel that containing 0.5% agarose (GenePure LE, ISC Bioexpress, Kaysville, UT, USA) and 1.0% Tris-barbital buffer, pH 8.6. Electrophoresis experiment was performed using an i-MyRun Horizontal Electrophoreis system (Cosmo Bio Co., Ltd., Tokyo, Japan.) in Tris-barbital buffer (0.2 M Tris-HCl (hydroxymethylamenomethane), 10 mM 5,5-diethylbarbituric acid, and 50 mM sodium diethyl barbiturate, pH 8.6), at 100V for 3 hours. After the electrophoresis, the agarose gel was stained with Coomassie brilliant blue G-250 solution (containing 0.1% Coomassie brilliant blue G-250 dye, 50% absolute methanol, 40% de-ionized water, and 10% glacial acetic acid) for 45 minutes and then de-stained with de-stain solution (containing 12% absolute methanol, 81% de-ionized water, and 7% glacial acetic acid). The relative electrophoretic mobility (REM) was

defined as the ratio of the distances that moved from the origin by Ox-LDL vs. native LDL.

3.5.3.7 Determination of Apo B-100 Fragmentation of LDL

The apo B-100 fragmentation of native or oxidized LDL was detected by SDS-PAGE according to the method described by Yoon *et al*. (66) with some modification. Ninety microliters of dialyzed LDL samples (final concentration at 300 μg LDL protein/ mL) were pre-incubated in microcentrifuge tubes with 5 μL of γ-OR (10, 100, 200, 300 μM final concentrations), α-tocopherol or ferulic acid solutions in DMSO (10 and 100 μM final concentrations) for 30 minutes at 37˚C. The *in vitro* oxidation of dialyzed LDL samples were initiated by adding 5 μL of freshly prepared aqueous $CuSO_4$ solution (30 μM final concentration) into LDL samples and then incubated at 37˚C for 4 hours in a VorTemp56™ shaker incubator (Labnet International, Inc., Woodbridge, NJ, USA). Individual LDL sample that incubated with DMSO and DDI instead of tested compounds and $CuSO_4$ solution was used as their own native LDL samples.

After 4 hours of incubation, 40 μL of samples were denatured with 40 μL of 4% SDS denaturation buffer solution of Laemmli (67) and then incubated at 95ºC for 5 minutes. For electrophoresis, 10 μL of each denatured samples were applied to SDS gel electrophoresis on 4% polyacrylamide gels (1 mm thick). Electrophoresis experiment was performed using an electrophoresis system (Power Supply-EPS 601, Hoefer™ Mighty Small II SE 260 (for 8x9 cm Gels), Hoefer™ Dual Gel Caster, and Image Scanner; Amersham Biosciences, Inc., Piscataway, NJ, USA) in a running buffer (0.025 M Tris-base, 0.192 M Glycine, and 0.1% SDS, pH 8.7), at 100V, 400mA for 2 hours. Prestained SDS-PAGE standard, broad range (Bio-Rad Laboratories, Inc., Cat. No. 161-0318) was also denatured and applied to electrophoresis in the same way as LDL samples. After the electrophoresis, the gel was stained with Coomassie brilliant blue G-250 solution (containing 0.1% Coomassie brilliant blue G-250 dye, 50% absolute methanol, 40% de-ionized water, and 10% glacial acetic acid) for 45 minutes and then de-stained with de-stain solution (containing 12% absolute methanol, 81% de-ionized water, and 7% glacial acetic acid). De-stained SDS gels were scanned with the Image Scanner (Amersham

Biosciences, Inc., Piscataway, NJ, USA), and the bands were measured using the Gel Documentation (Gel Doc) and image analysis systems (G: Box Chemi SD model and GeneSnap software, version 7.01; SynGene, Synoptics Ltd., Cambridge, England).

3.6 Statistical Analysis of Data

Each experiment was repeated to provide a minimum of three independent replications. All the data from the experiments were expressed as mean ± SEM. Statistical analysis of data were performed by using SPSS 11.5 software (SPSS Inc., Chicago, IL, USA) for Windows. Statistical significances were determined using paired-t test to compare means between the Ox-LDL samples pre-incubated with tested compounds; γ-OR, ferulic acid, or α-tocopherol at various concentrations and ones without pre-incubation in TBARS experiment. One-way ANOVA and Tukey HSD test was performed to compare means of parameters within each treatment; γ-OR, ferulic acid, α-tocopherol, or cholesterol at various concentrations as well as within each concentration of treatments. Significant differences were considered at $p\text{-value} < 0.05$.

3.7 Ethical Consideration

Ethical consideration was not needed for the following experiments; γ-OR components analysis, the bioaccessibility and bioavailability of γ-OR, and the effect of γ-OR on the cholesterol micellarization and cholesterol uptake using Caco-2 cell culture.

The HMG-CoA reductase activity experiment part of this study, animal facilities and protocol were approved by the Laboratory Animal Care and Use Committee at Faculty of Veterinary Science, Chulalongkorn University, Thailand.

The LDL-oxidation part of this study was approved and conducted according to the requirements of the Ethical Committee of Health Science, Chulalongkorn University, Thailand. Written informed consents from the volunteers were obtained before blood collection.

CHAPTER IV

RESULTS

4.1 Extraction and Identification of γ-OR in Samples by HPLC

Gamma-oryzanol was analyzed using reverse phase HPLC coupled with a photo diode array detector and determined at wavelength 325 nm. Ten components of the γ-OR standard were identified as Δ^7-stigmastenyl ferulate, stigmasteryl ferulate, cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, Δ^7-campestenyl ferulate, campesteryl ferulate, Δ^7-sitostenyl ferulate, sitosteryl ferulate, campestanyl ferulate, and sitostanyl ferulate at the retention time as shown in Figure 4.1. Each peak of γ-OR components was also confirmed by its unique maximum absorbance spectrum of ferulic acid at wavelength 324-325 nm. Cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, campesteryl ferulate, and sitosteryl ferulate were the four major components found in the γ-OR standard approximately 32%, 38%, 17%, and 9%, respectively. It was also found that those four components dominated in chyme and filtered aqueous (micelles) fractions from the digested meal during simulated digestion (Figure 4.2).

The γ-OR standard was spiked into the samples for recovery test. It showed 95.5% recovery of spiked γ-OR standard. The limit of γ-OR detection in this study was at the concentration of γ-OR less than 0.002 mg/mL which six minor components of γ-OR including Δ^7-stigmastenyl ferulate, stigmasteryl ferulate, Δ^7-campestenyl ferulate, Δ^7-sitostenyl ferulate, campestanyl ferulate, and sitostanyl ferulate still could be detected.

4.2 Bioavailability and Bioaccessibility of γ-OR

4.2.1 Bioaccessibility of γ-OR Using a Simulated Digestion Method

After simulated digestion, chyme and micelles fraction were extracted and analyzed for γ-OR components using reverse phase-HPLC method (3). The HPLC-PAD 3D plot profiles of A, B, C, and D in Figure 4.3 represented the γ-OR components in the extracts of chyme and micelles fraction from digested rice meal containing peanut oil

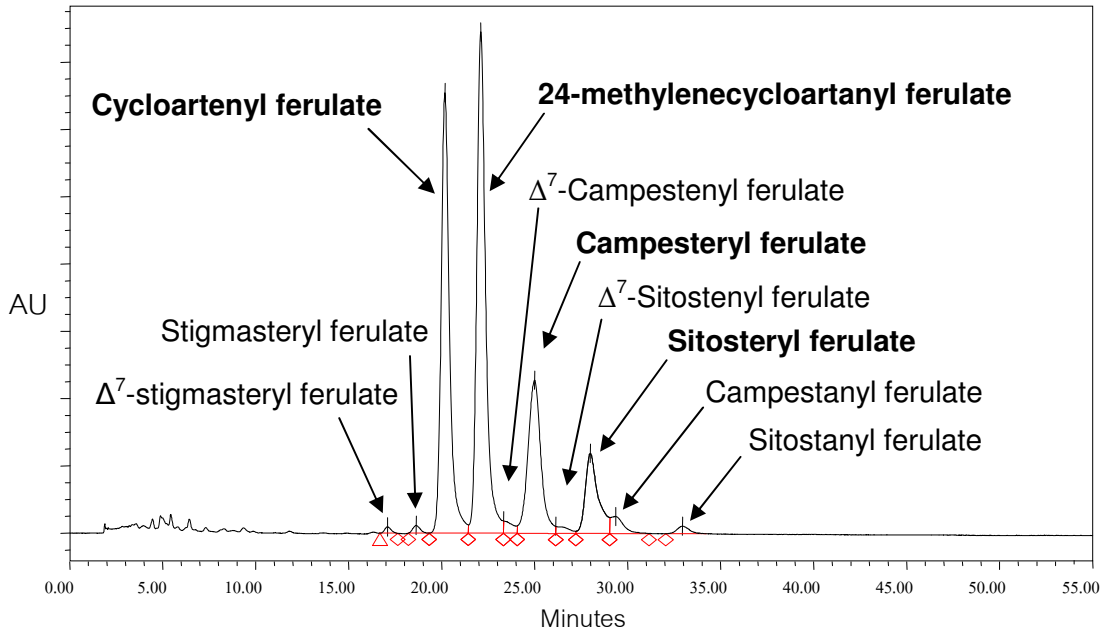


Figure 4.1 A profile of γ-OR standard determined by HPLC-PAD at wavelength 325 nm

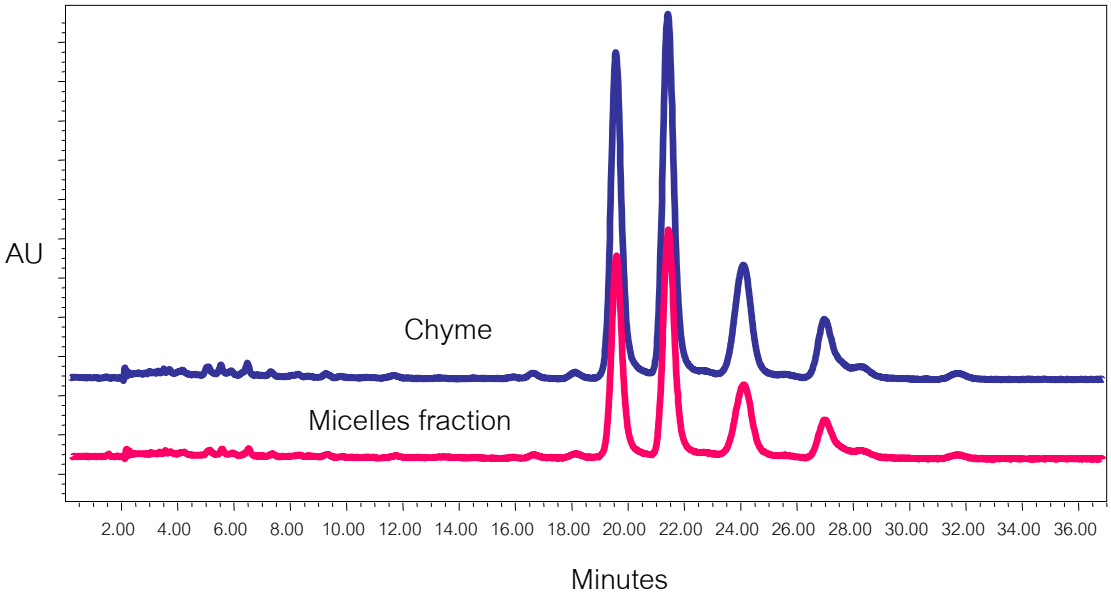


Figure 4.2 Representative HPLC-PAD profiles of γ-OR in chyme and micelles fraction after the simulated digestion of rice meal containing γ-OR rich peanut oil

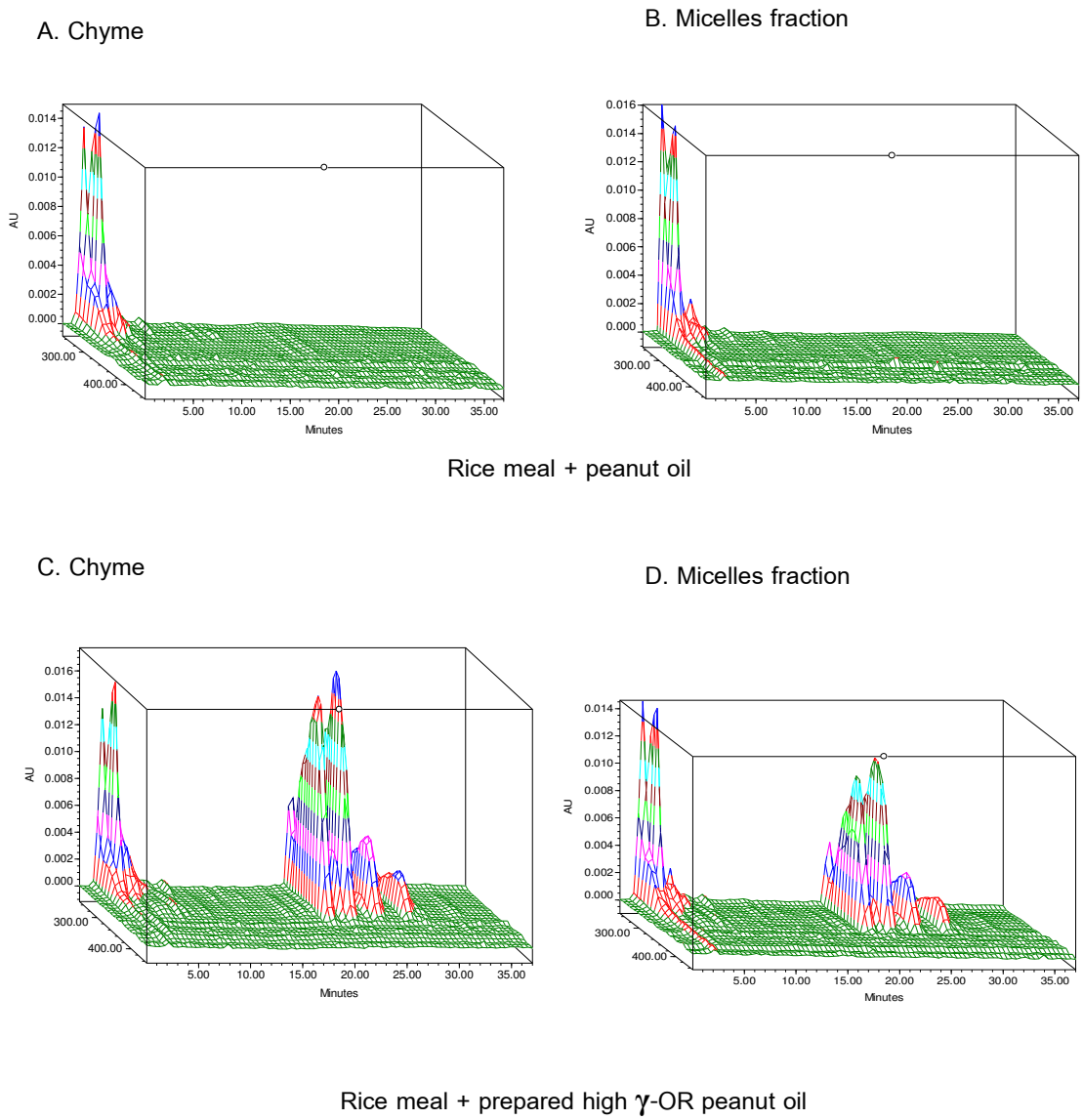


Figure 4.3 HPLC-PAD profiles (3D plot) of chyme (A, C) and micelles fraction (B, D) of rice meal containing peanut oil or high γ-OR peanut oil after simulated digestion

alone and from those containing high γ-OR peanut oil, respectively. It was obvious that peanut oil and rice meal used in this study did not contain γ-OR even in chyme or micelle fraction after simulated digestion. Whereas rice meal mixed with prepared high γ-OR peanut oil clearly showed a γ-OR component profile in chyme and micelle fraction after simulated digestion.

Bioacccessibility of γ-OR was shown in Figure 4.4 as the efficiency of γ-OR component micellarization during the simulated digestion. There were no significant differences in the efficiency of micellirization among ten γ-OR components ($p > 0.05$). More than 50% of each γ-OR component were incorporated into the micelle fraction (Figure 4.4). Cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, campesteryl ferulate, and sitosteryl ferulate were four major components of γ-OR incorporated into the micelle fraction and presented apprimately 31.2%, 37.2%, 17.0%, and 8.9% of total amount of γ-OR incorporated, respectively (Figure 4.5). In addition, recoveries of γ-OR components after the simulated oral, gastric, and small intestinal phases of digestion exceeded 80% and were not significantly different ($p > 0.05$). This result suggested that intact γ-OR can be well incorporated into mixed micelles before absorption by small intestine epithelial cells.

4.2.2 Bioavailability of γ-OR

Bioavailability of γ-OR was determined using simulated digestion coupled with a Caco-2 cell culture method. The cellular uptake of micellarized γ-OR was determined. Caco-2 cell monolayers were used at 11-14 days after reaching 100% confluent. The characteristic of this cell line at this stage were like enterocytes-like cells or small intestine epithelial cells and showed specific morphology of apical brush border surfaces as domes on the monolayers that could be clearly seen with microscope magnification. Figure 4.6 showed the examples of domes on the differentiated Caco-2 cells at 12 days after monolayer reached 100% confluent. The intact Caco-2 cell monolayers were observed in the cellular uptake experiments.

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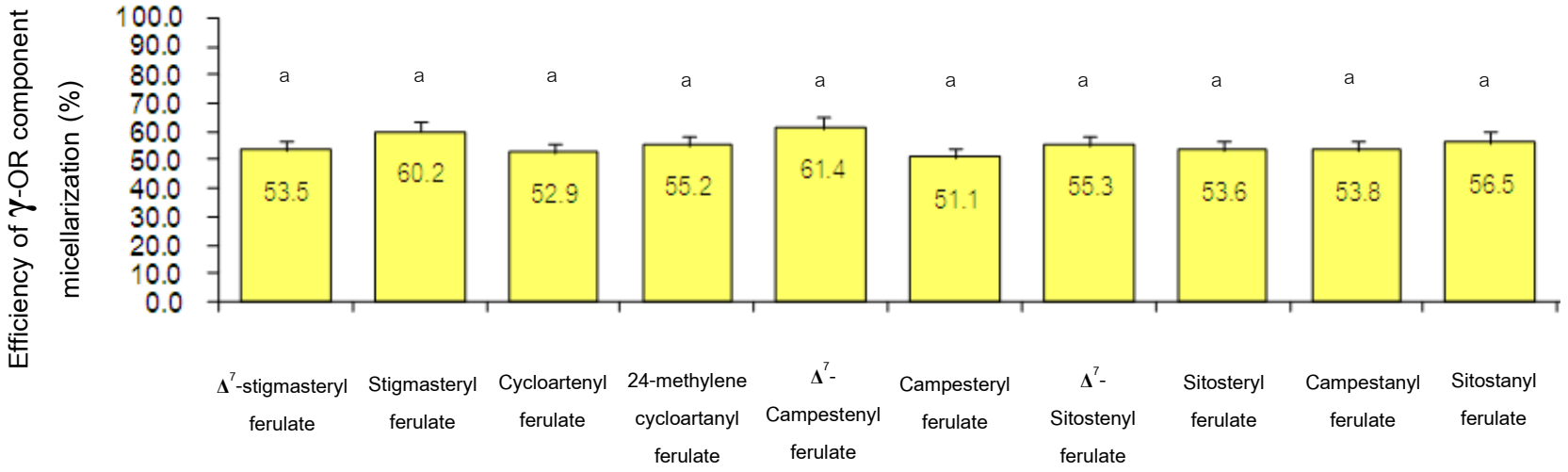


[a] *Same letter indicates no significant difference among means of each γ-OR component*

Figure 4.4 Bioaccessibility of γ-OR during the simulated digestion (means ± SEM, n = 5)

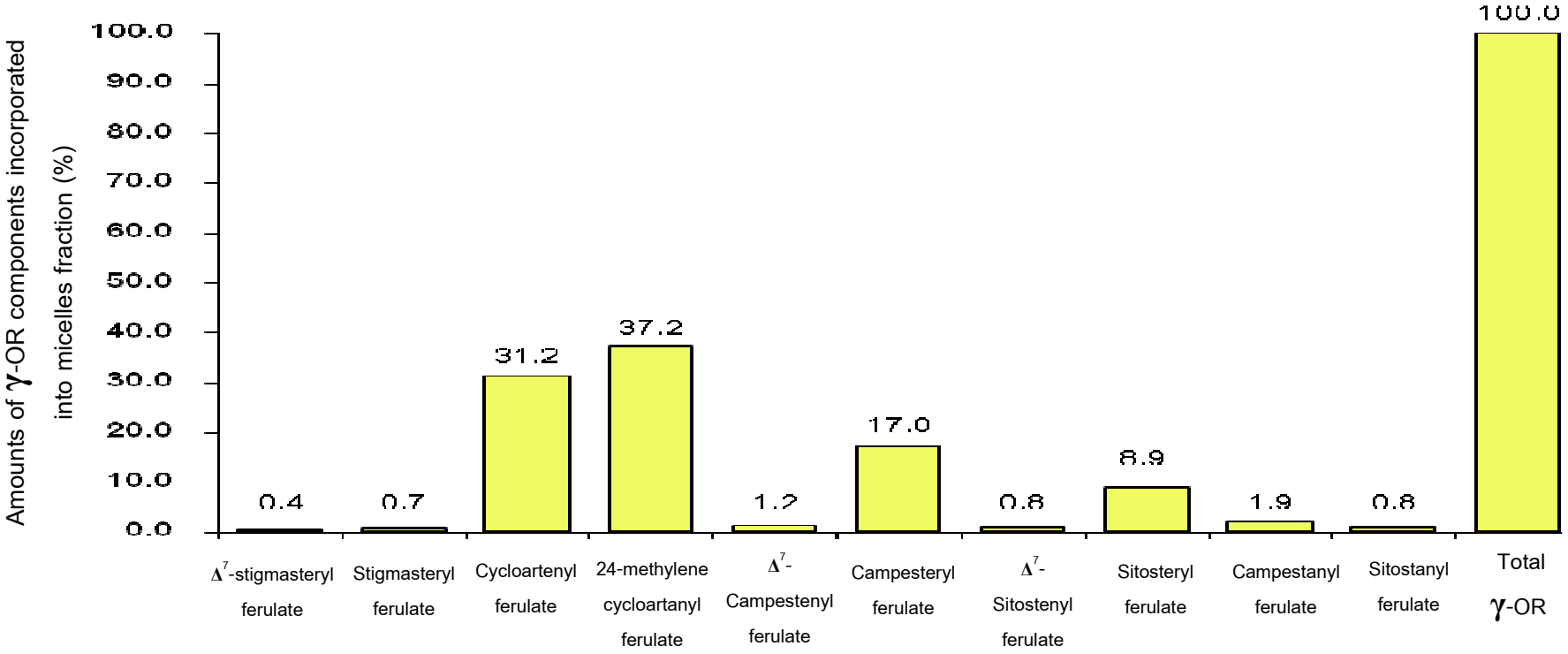
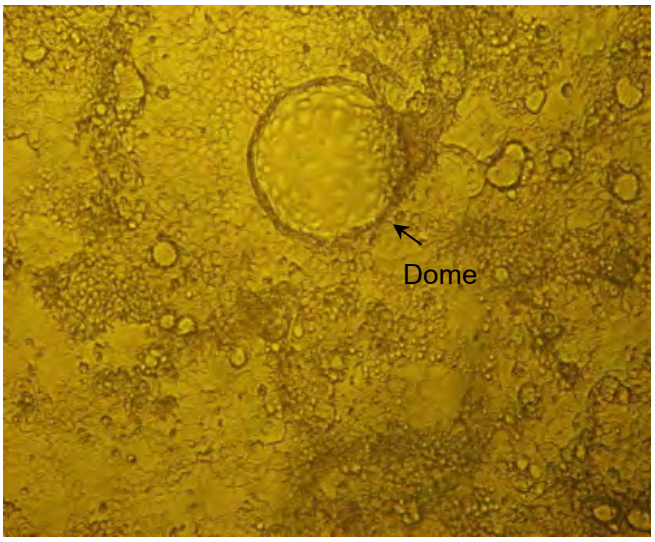
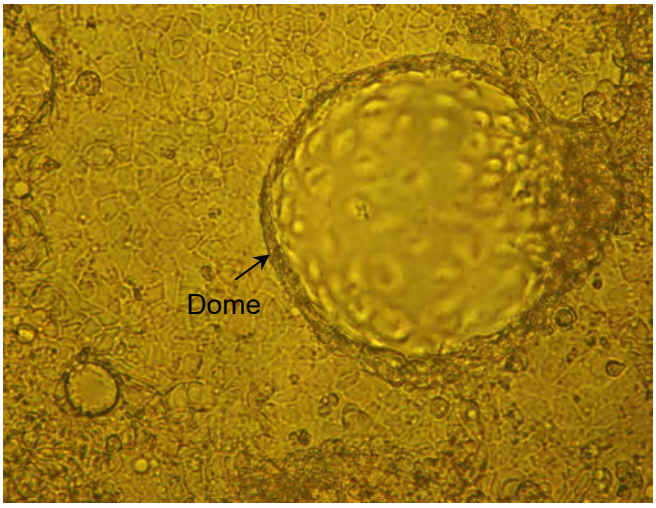


Figure 4.5 Percent abundance of γ-OR components incorporated into micelles during the simulated digestion (means ± SEM, n = 5)

In the cellular uptake of micellarized γ-OR experiment, monolayers of Caco-2 cell culture were microscopically observed during incubation period. It has been shown in Figure 4.7 that micellarized γ-OR fraction from the simulated digestion did not affect the intact of Caco-2 cell monolayers during the indicated incubation time. The result showed that micellarized γ-OR was poorly uptaken by differentiated Caco-2 intestinal cells. Only 2.46 ± 0.16 % (mean ± SEM) of total γ-OR per mg of cells protein could be absorbed by Caco-2 intestinal cells.

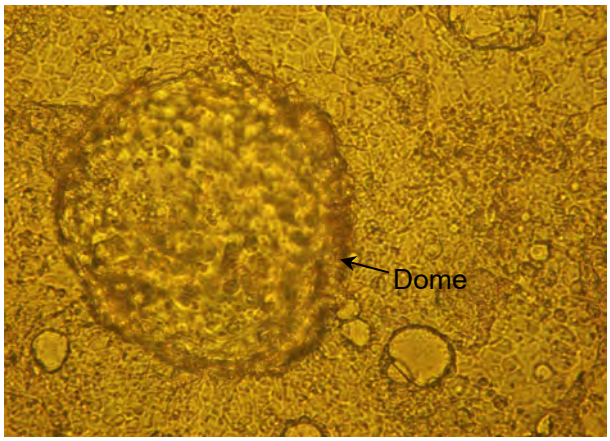


A. *Microscopic magnified 10X*

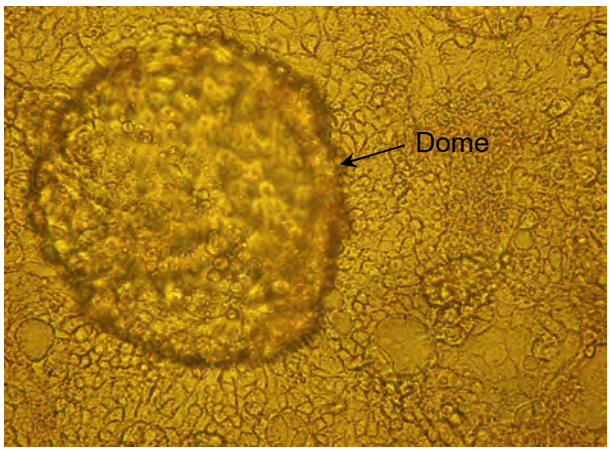


B. *Microscopic magnified 20X*

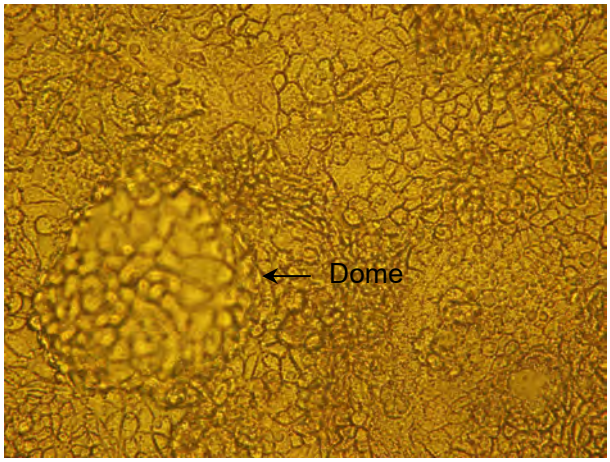
Figure 4.6 Differentiated Caco-2 cell culture (ATCC HTB-37) at 12 days after reaching 100% confluent magnified by Olympus IX50 microscope



A. *Microscopic magnified 40X, after 2 hour incubation with micellarized γ-OR*



B. *Microscopic magnified 40X, after 3 hour incubation with micellarized γ-OR*



C. *Microscopic magnified 40X (different focus), after 4 hour incubation with micellarized γ-OR*

Figure 4.7 Microscopic observation of treated Caco-2 cell culture during incubation with micellarized γ-OR magnified by the Olympus IX50 microscope

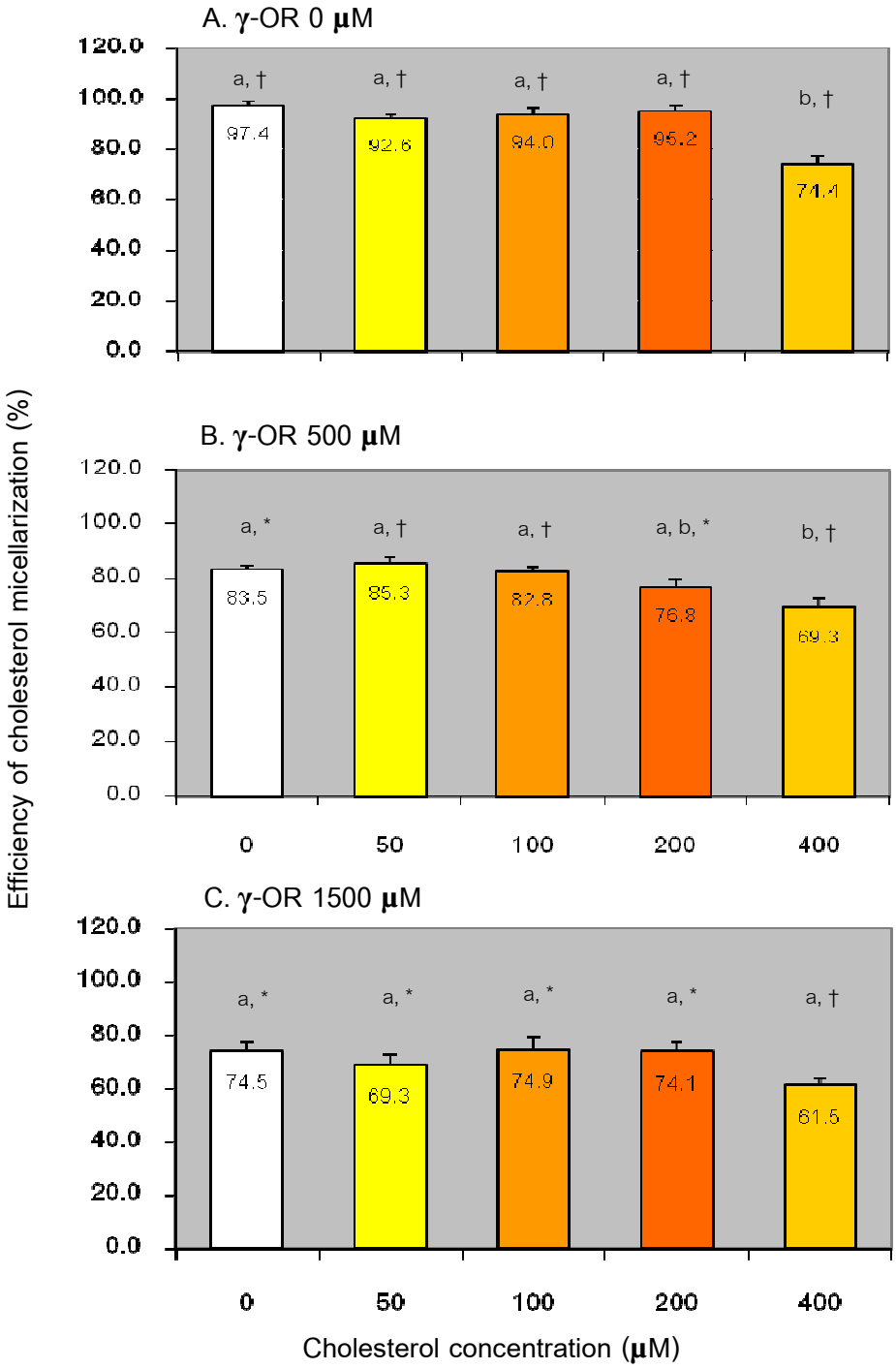
4.3 Effect of γ-OR on the Micellarization and Uptake of Cholesterol

4.3.1 Effect of γ-OR on the Micellarization of Cholesterol – Synthetic Micelles with ^{14}C-Cholesterol

Synthetic micelles with ^{14}C-cholesterol preparation at various concentrations of total cholesterol and γ-OR were conducted to investigate the effect of γ-OR on the micellarization of cholesterol. The amounts of ^{14}C-cholesterol in pre-centrifuged solution represented as synthetic chyme and in micelle fraction (supernatant after centrifugation) were determined. The efficiency of cholesterol incorporated into synthetic micelles were calculated from ^{14}C-cholesterol values in micelle fraction and in pre-centrifuged solution, assuming that ^{14}C-cholesterol traces represent total cholesterol in the system.

Because of very little amount of ^{14}C-Cholesterol traces used in this experiment, it was not counted in calculated cholesterol. In Figure 4.8A indicated that under this condition, cholesterol concentrations of 50-200 μM had no significant effect on efficiency of cholesterol incorporation into synthetic micelles. High concentration of cholesterol at 400 μM tended to decrease in its own efficiency to incorporate into synthetic micelles. Comparing among the same treatment with various concentrations of cholesterol, a decrease in the efficiency of cholesterol incorporation into synthetic micelles was observed only in samples treated with 500 μM γ-OR in the presence of 400 μM of cholesterol ($p<0.05$). The presence of γ-OR at 500 μM did not lower the efficiency of cholesterol micellarization at 50, 100, and 200 μM of cholesterol (Figure 4.8B).

When considering the control groups with absence of cholesterol in each treatment, it was found that γ-OR at both concentratons (500 and 1500 μM) significantly affected the efficiency of cholesterol incorporation into synthetic micelles by lowering baseline ($p<0.05$). The significant decrease in the efficiency of cholesterol micellarization in the γ-OR group at concentration of 1500 μM was observed when cholesterol concentration was less than 400 μM ($p<0.05$) (Figure 4.8A-C).



[a b] *Different letters indicate significantly different means among γ-OR at the same concentration*

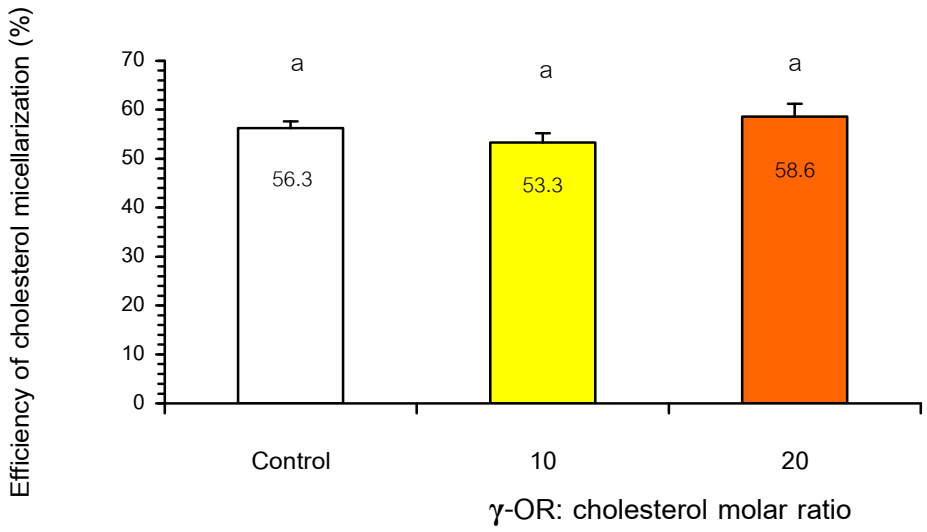
[* †] *Different symbols indicate significantly different means among cholesterol at the same concentration*

Figure 4.8 Efficiency of cholesterol micellarization into synthetic micelles at various concentrations of γ-OR (mean ± SEM, n = 5)

4.3.2 Effect of γ-OR on the Micellarization of Cholesterol - Simulated Digestion with ^{14}C-Cholesterol

In addition to the synthetic micelle preparation, a simulated digestion method with ^{14}C-cholesterol was also conducted to affirm the effect of γ-OR on the micellarization of cholesterol. Rice meals mixed with peanut oil containing 0- (control), 10- and 20-fold molar ratio of γ-OR to cholesterol with ^{14}C-cholesterol traces (1 μCi) were digested. ^{14}C-cholesterol traces accounted about 30% of normal cholesterol in each reaction. Determined ^{14}C-cholesterol in chyme and micelle fraction was used to represent total cholesterol for calculating the efficiency of cholesterol incorporated into the mixed micelles during simulated digestion.

Effect of γ-OR on the efficiency of cholesterol micellarization during simulated digestion was shown in Figure 4.9. Presence of γ-OR in both of 10- and 20- fold molar ratio to cholesterol had no effect on the efficiency of cholesterol micellarization compared with the control group ($p > 0.05$).

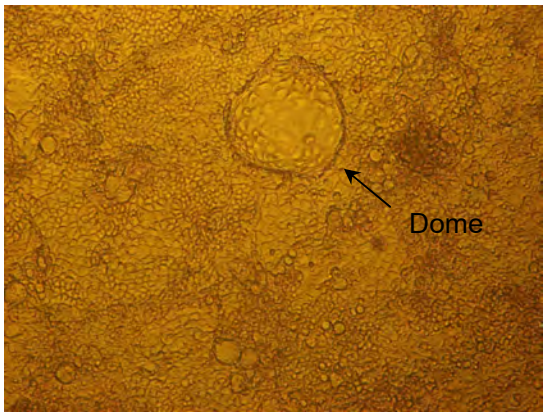


[a] *Same letter indicates no significant difference among means of treaments*

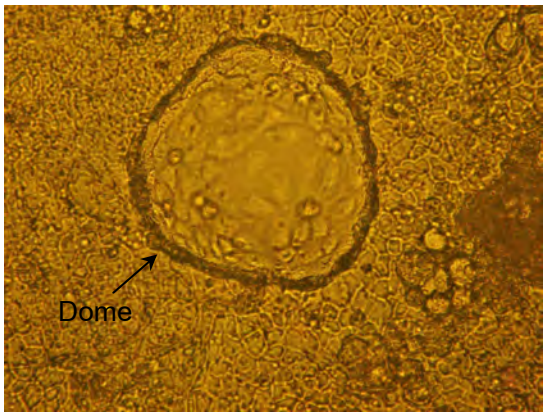
Figure 4.9 Efficiency of cholesterol micellarization during simulated digestion with the absence or presence of γ-OR (means ± SEM, n = 3)

4.3.3 Effect of γ-OR on the Cellular Uptake of Cholesterol - Simulated Digestion with ^{14}C-Cholesterol and Coupled with Caco-2 Cell Culture

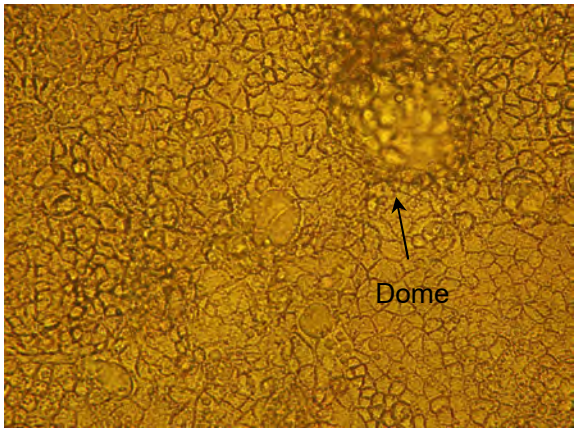
The effect of γ-OR on the cellular uptake of cholesterol was determined using simulated digestion coupled with Caco-2 cell culture method. The cellular uptake of micellarized cholesterol with the absence or presence of γ-OR was determined. The intact monolayers of differentiated Caco-2 cells were observed before micelle treatments in the cellular uptake experiments. It was that micelle fraction from the simulated digestion did not affect the intact of Caco-2 cell monolayers during the indicated incubation time (Figure 4.10).



A. *Microscopic magnified 10X, after 2 hour incubation with micellarized γ-OR and cholesterol*

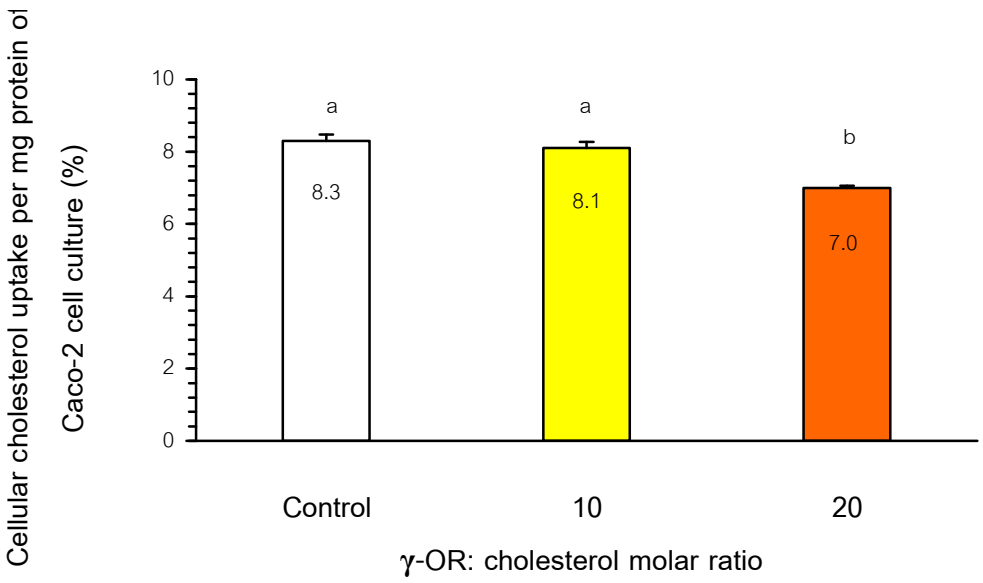


B. *Microscopic magnified 40X, after 3 hour incubation with micellarized γ-OR and cholesterol*



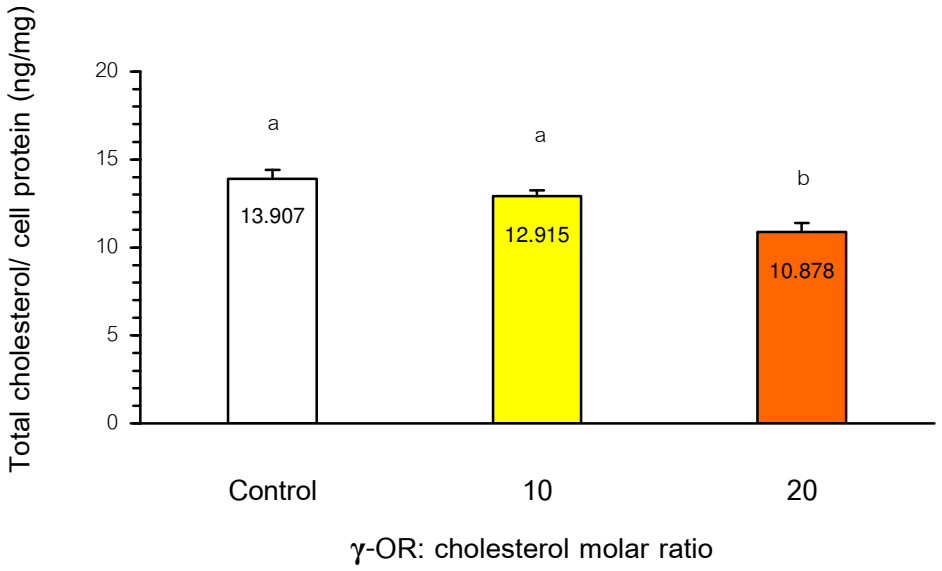
C. *Microscopic magnified 40X (different focus), after 4 hour incubation with micellarized γ-OR and cholesterol*

Figure 4.10 Microscopic observation of the example of treated Caco-2 cell culture during incubation period with micellarized γ-OR and cholesterol, magnified by Olympus IX50 microscope



[a b] Different letters indicate significantly different means among treaments

Figure 4.11 Percentage of cholesterol uptake by Caco-2 cells after treatment with micelle fractions from simulated digestion of cholesterol with the absence or presence of γ-OR for 4 hours (means ± SEM, n = 3)



[a b] Different letters indicate significantly different means among treaments

Figure 4.12 Accumulated total cholesterol in Caco-2 cells (ng/ mg cell protein) after treatment with micelle fraction from simulated digestion of cholesterol with the absence or presence of γ-OR for 4 hours (means ± SEM, n = 3)

Effect of γ-OR on the cellular uptake of cholesterol after simulated digestion was shown in Figure 4.11 and Figure 4.12. It was found that the presence of γ-OR at 20- fold molar ratio to cholesterol significantly decreased the cholesterol uptake by Caco-2 intestinal cells, compared with the control group ($p < 0.01$). Whereas γ-OR at 10- fold molar ratio to cholesterol did not affect the cellular cholesterol uptake ($p > 0.05$).

The results in Phase III suggested that γ-OR at high molar ratio to cholesterol had the inhibition activity to cholesterol absorption by enterocytes even though at the same molar ratio did not decrease the efficiency of cholesterol micellarization during digestion.

4.4 Effects of γ-OR and ferulic acid on the HMG-CoA reductase activity

The effects of γ-OR (0, 100, 200, and 400 μM) and ferulic acid (0, 100, 200, and 400 μM) on the HMG-CoA reductase activity were determined and compared with simvastatin (239 μM) as a positive control.

The result showed that both γ-OR and ferulic acid had inhibitory effect on HMG-CoA reductase activity beginning at the lowest concentration (100 μM) (Figure 4.13). Based on the trend lines, at the same concentration (239 μM), simvastatin seemed to be the weakest inhibitory effect on HMG-CoA reductase activity (42.9 ± 4.5 %), compared with those of γ-OR (~50%), and those of ferulic acid (~60%) (Figure 4.14). On the other hand, ferulic acid seemed to be the strongest HMG-CoA reductase inhibitor compared with γ-OR and simvastatin in this study.

4.5 Antioxidant activities of γ-OR against LDL oxidation

4.5.1 Characteristic Data of Volunteers

Fifteen healthy male volunteers were recruited under the previously described inclusion criteria for the experiments in this phase. However, isolated LDL fraction from collected blood of one volunteer was not adequate to use for all antioxidant experiments in Phase V. Therefore, only 7, 10, 6, and 3 of LDL samples were used in the conjugated

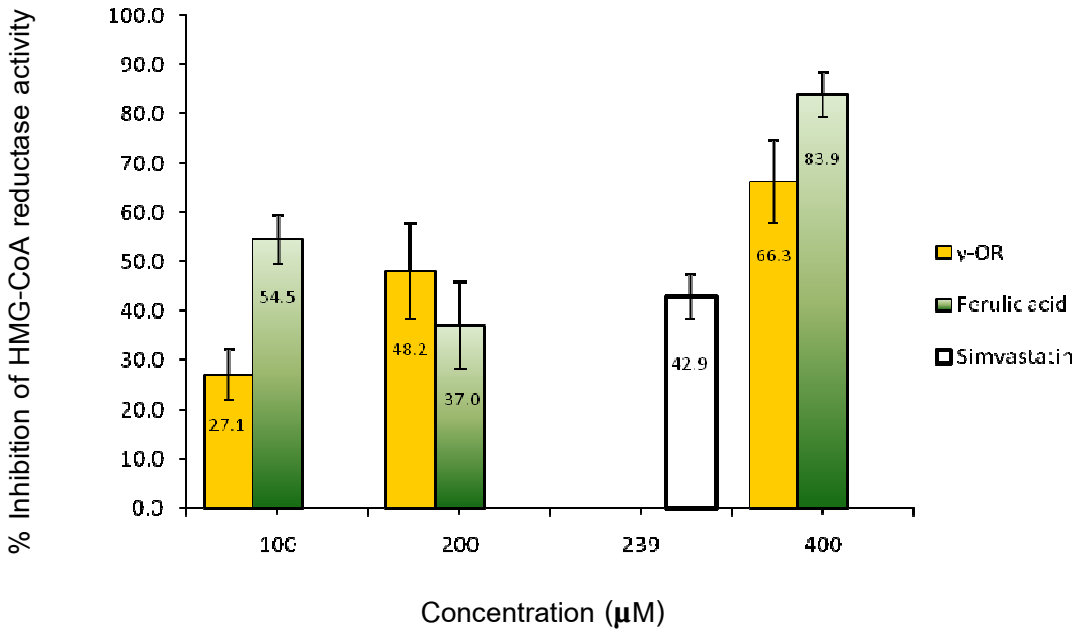


Figure 4.13 Inhibitory effects of γ-OR, ferulic acid, and simvastatin on HMG-CoA reductase activity (mean ± SEM, n = 4)

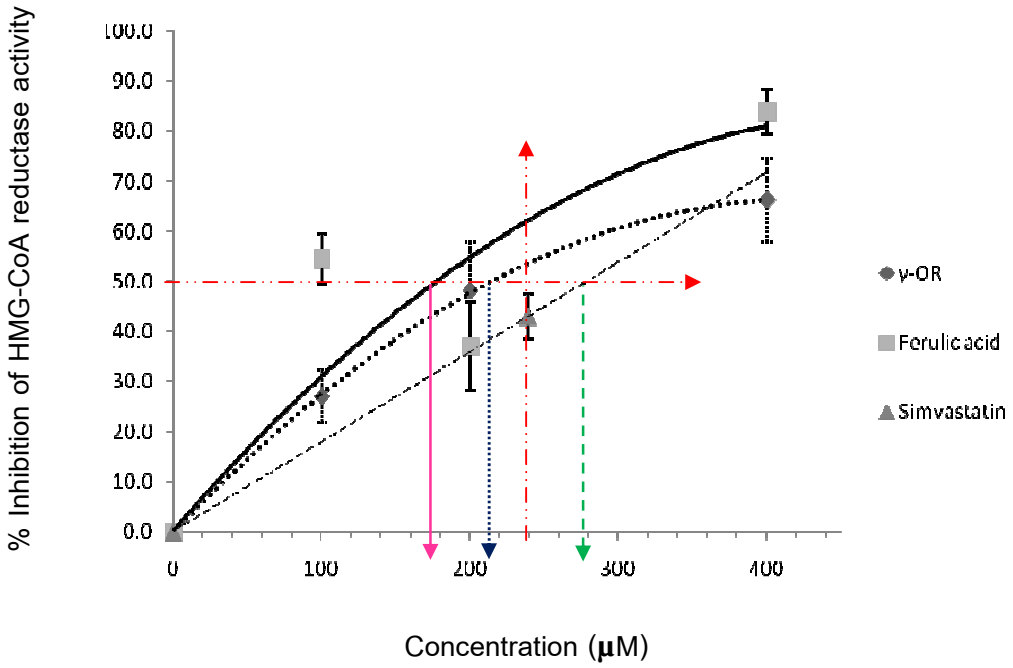


Figure 4.14 Inhibitory effects of γ-OR, ferulic acid, and simvastatin on HMG-CoA reductase activity with trend lines (mean ± SEM, n = 4)

dienes, TBARS, relative electrophoretic mobility, and apo B-100 fragmentation experiments, respectively. Characteristic data of volunteers were shown in Table 4.1.

Table 4.1 Characteristic data of volunteers

Parameters	Value*
Age (years)	22.1 ± 0.8
Weight (kg)	66.1 ± 2.2
Height (m)	1.70 ± 0.02
BMI (kg/m^2)	22.9 ± 0.7
Systolic blood pressure (mm-Hg)	114.1 ± 2.6
Diastolic blood pressure (mm-Hg)	66.9 ± 1.9
Fasting blood glucose (mg/ dL)	82.9 ± 1.1
Plasma total cholesterol (mg/ dL)	179.1 ± 8.1
Plasma triacylglycerol (mg/ dL)	57.1 ± 3.9
Plasma HDL (mg/ dL)	44.9 ± 2.1
Plasma LDL (mg/ dL)	122.8 ± 7.8
Aspartate Aminotransferase (AST) (U/ L)	27.4 ± 5.9
Alanine Aminotransferase (ALT) (U/ L)	20.5 ± 3.8
Alkaline phosphatase (U/ L)	57.3 ± 3.8

*** Mean ± SEM, n=15**

4.5.2 Conjugated Dienes Determination of *in vitro* Oxidized LDL

The effect of γ-OR, ferulic acid, and α-tocopherol at various concentrations on conjugated diene formation during Cu^{2+}-induced LDL oxidatiod monitored by measuring the conjugated diene formation at a wavelength of 234 nm for 5 hours (Table 4.2 and 4.3). Conjugated dienes formation referred to the peroxidation of lipids, such as cholesterol, triacyglycerol, and fatty acids, in LDL samples. The formation demonstrated by lag time, % prolongation from lag time and decomposition time of conjugated dienes formation, a propagation rate, and a concentration of conjugated dienes formation or conjugate dienes production at decomposition time. The measurement of each

parameter of conjugated dienes formation as the kinetics of LDL oxidation of a basal Ox-LDL sample from this study was shown in Figure 4.15.

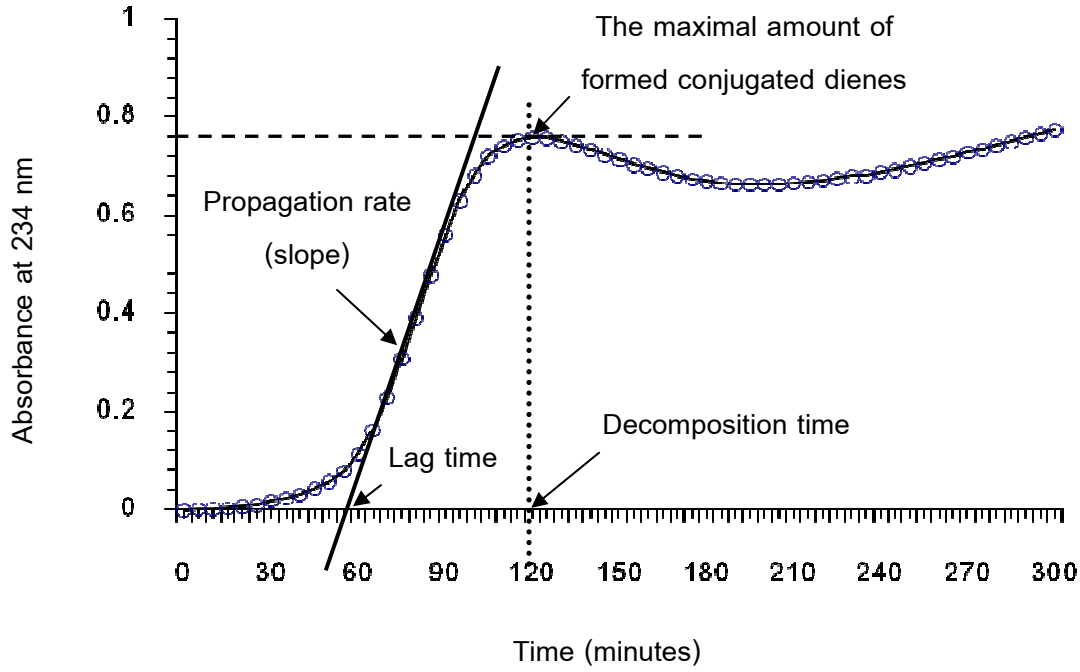


Figure 4.15 Kinetics of LDL oxidation as determined by measuring parameters of the change in absorbance at wavelength 234 nm

From the conjugated dienes formation monitoring, the results showed that all of γ-OR, ferulic acid, and α-tocopherol had antioxidative activities against Cu^{2+} induced LDL oxidation and their activities were dosely dependent. Higher concentrations of those compounds prolonged the lag time and the decomposition time of conjugated dienes formation in Ox-LDL. Percentage of lag time prolongation was increased by the concentrations of antioxidants. Whereas, the propagation rate and the conjugated dienes production at decomposition time during LDL oxidation were decreased.

Ferulic acid at the concentration of 10 μM, dramatically prolonged lag time of conjugated dienes formation to triple times as much as that of control Ox-LDL (179.1 ± 29.9 vs. 67.8 ± 8.0 minutes, respectively) (p = 0.002) whereas γ-OR presented its effect at the concentration of 50 μM (236.3 ± 16.7 minutes) (p < 0.001) (Table 4.2). Nevertheless, α-tocopherol had the stronger antioxidant activity than γ-OR and ferulic acid had on the prolongation of lag time of conjugated dienes during LDL oxidation at low concentration. It significantly prolonged the lag time of conjugated dienes formation

at 5 μM (130.0 ± 16.4 minutes, p = 0.014). Also its activity significantly increased at higher concentrations of 10 μM (145.8 ± 16.2 minutes, p = 0.038) and 50 μM (258.2 ± 8.4 minutes, $p < 0.001$) (Table 4.2).

At concentration of 1 μM, γ-OR, ferulic acid, and α-tocopherol did not show remarkable increase in % prolongation of lag time during LDL oxidation, compared with control Ox-LDL ($p > 0.05$) (Table 4.2). At concentration of 5 μM, α-tocopherol showed a significant increase in % prolongation of lag time compared with control Ox-LDL (94.9 ± 16.4 vs. 0 %, p = 0.045). Ferulic acid showed an increase in % prolongation of lag time but no significant difference from control at the concentration of 5 μM (72.1 ± 44.0 %, $p > 0.05$) but showed a significant increase in that at the concentration of 10 μM (204.5 ± 73.4 %, p = 0.005). All test compounds at concentration of 50 μM showed significant increase in % prolongation ($p < 0.001$).

When considering the ability on prolonging the decompostion time of conjugate diene formation among treatments including three compounds at concentration of 10 μM and control Ox-LDL, it was found that only ferulic acid showed a significant prolongation (Table 4.2). Nevertheless, at the concentration of 50 μM, all of γ-OR, ferulic acid, and α-tocopherol showed markedly prolongation on the decomposition time of conjugated dienes formation (262.0 ± 8.6, 267.5 ± 11.6, and 262.5 ± 10.3 minutes, respectively).

To prolong the propagation rate of conjugated dienes formation, ferulic acid at 10 μM and 50 μM significantly decreased the propagation rates (p = 0.004 and < 0.001, respectively) whereas γ-OR and α-tocopherol presented at concentration of 50 μM (p = 0.002 and 0.003, respectively) (Table 4.3). All of γ-OR, ferulic acid, and α-tocopherol at concentration of 50 μM significantly decreased the concentration of produced conjugated dienes during LDL oxidation ($p < 0.001$, < 0.001, and 0.001, respectively) (Table 4.3).

Table 4.2 Lag time, % prolongation from lag time, and decomposition time of conjugated dienes formation during LDL oxidation with various concentrations of γ-OR, ferulic acid, and α-tocopherol

Ox-LDL samples[1]	Lag time (minutes)	% prolongation from lag time	Decomposition time (minutes)
DMSO (control Ox-LDL)	67.8 ± 8.0 $^{a, c, e, g, †, ‡, π}$	0 $^{a, c, e, g, †, ‡, π}$	163.2 ± 24.4 $^{a, c, e, g, †, ‡, π}$
γ-OR 1 μM	77.3 ± 10.1 $^{a, †}$	15.9 ± 11.2 $^{a, †, *}$	178.0 ± 30.8 $^{a, †}$
γ-OR 5 μM	74.9 ± 8.3 $^{c, †}$	13.2 ± 9.3 $^{c, d, †}$	186.0 ± 27.4 $^{c, †}$
γ-OR 10 μM	117.5 ± 15.8 $^{e, f, †}$	73.7 ± 13.2 $^{e, f, *}$	207.4 ± 23.2 $^{e, f, †}$
γ-OR 50 μM	236.3 ± 16.7 ^{h,*}	261.3 ± 25.5 $^{h, #}$	262.0 ± 8.6 $^{h, †}$
Ferulic acid 1 μM	64.1 ± 7.2 $^{a, ‡}$	-2.4 ± 9.7 $^{a, ‡}$	165.2 ± 28.4 $^{a, ‡}$
Ferulic acid 5 μM	101.0 ± 17.3 $^{c, d, ‡}$	72.1 ± 44.0 $^{c, d, ‡, ω}$	213.7 ± 18.8 $^{c, ‡, &}$
Ferulic acid 10 μM	179.1 ± 29.9 $^{f, &}$	204.5 ± 73.4 $^{f, &, ω}$	261.3 ± 6.4 $^{f, &}$
Ferulic acid 50 μM	267.5 ± 11.6 $^{h, ω}$	321.9 ± 44.9 $^{h, &}$	267.5 ± 11.6 $^{h, &}$
α-tocopherol 1 μM	95.8 ± 10.5 $^{a, π, α}$	43.2 ± 9.0 $^{b, π, α}$	200.2 ± 28.1 $^{a, π, α}$
α-tocopherol 5 μM	130.0 ± 16.4 $^{d, α}$	94.9 ± 16.4 $^{d, α}$	214.1 ± 23.0 $^{c, π, α}$
α-tocopherol 10 μM	145.8 ± 16.2 $^{f, α}$	119.9 ± 17.3 $^{e, f, α}$	226.8 ± 17.5 $^{e, f, π, α}$
α-tocopherol 50 μM	258.2 ± 8.4 $^{h, β}$	305.2 ± 38.2 $^{h, β}$	262.5 ± 10.3 $^{h, α}$

Values express mean ±SEM, [1] 50 μg protein/mL, incubated with $CuSO_4$ 10 μM, 37°C, n =7

$^{† ‡ π * # & ω α β}$ *Different symbols indicate significantly different means within each treatment (including control)*

$^{a b c d e f g h}$ *Different letters indicate significantly different means within each concentration (including control)*

Table 4.3 Propagation rate and the conjugated dienes production at decomposition time during LDL oxidation with various concentrations of γ-OR, ferulic acid, and α-tocopherol

Ox-LDL samples[1]	Propagation rate (nmol· min^{-1}· g^{-1} of LDL protein)	conjugated dienes production (nmol·g^{-1} of LDL protein)
DMSO (control Ox-LDL)	3.92 ± 0.77 $^{a, c, e, g, †, ‡, π}$	319.47 ± 48.24 $^{a, c, e, g, †, ‡, π}$
γ-OR 1 μM	3.35 ± 1.13 $^{a, †, *}$	291.17 ± 70.06 $^{a, †}$
γ-OR 5 μM	3.23 ± 0.76 $^{c, †, *}$	295.67 ± 38.39 $^{c, †}$
γ-OR 10 μM	2.96 ± 0.67 $^{e, †, *}$	292.83 ± 45.53 $^{e, †}$
γ-OR 50 μM	0.63 ± 0.36 $^{h, *}$	65.25 ± 26.94 $^{h, *}$
Ferulic acid 1 μM	4.15 ± 1.08 $^{a, ‡}$	319.01 ± 57.76 $^{a, ‡}$
Ferulic acid 5 μM	1.97 ± 0.66 $^{c, ‡, &}$	267.93 ± 40.76 $^{c, ‡}$
Ferulic acid 10 μM	0.70 ± 0.23 $^{f, &}$	143.89 ± 56.78 $^{e, ‡, &}$
Ferulic acid 50 μM	0.01 ± 0.01 $^{h, &}$	12.25 ± 9.13 $^{h, &}$
α-tocopherol 1 μM	3.81 ± 0.98 $^{a, π}$	332.06 ± 54.28 $^{a, π}$
α-tocopherol 5 μM	3.16 ± 0.80 $^{c, π}$	288.72 ± 49.85 $^{c, π, α}$
α-tocopherol 10 μM	2.18 ± 0.56 $^{e, π}$	214.21 ± 61.34 $^{e, π, α}$
α-tocopherol 50 μM	0.82 ± 0.70 $^{h, π}$	84.02 ± 49.79 $^{h, α}$

Values express mean ±SEM, [1] 50 μg protein/mL, incubated with $CuSO_4$ 10 μM, 37°C, n =7

$^{† ‡ π * & α}$ *Different symbols indicate significantly different means within each treatment (including control)*

$^{a b c d e f g h}$ *Different letters indicate significantly different means within each concentration (including control)*

4.5.3 TBARS Determination of *in vitro* Oxidized LDL

After the propagation phase of LDL oxidation monitored by conjugated dienes formation, it was followed by the decomposition or degradation phase, which the cleavage of double bonds and the formation of aldehydes, such as MDA, occurred. MDA and aldehyde substances could be determined by TBARS assay.

TBARS contents in native and Ox-LDL were measured after initiated the *in vitro* oxidation of dialyzed LDL samples by 30 μM $CuSO_4$ (final concentration) and incubated at 60°C for 4 hours. Individual LDL sample that incubated with DMSO and DDI instead of tested compounds and $CuSO_4$ solution was used as its own native LDL sample.

Autooxidation of dialyzed LDL (native LDL) was shown as a small amount of TBARS in LDL samples that did not incubated with $CuSO_4$ (2.04 ± 0.70 vs. 2.11 ± 0.76 nmol MDA/ mg LDL protein in PBS and in DMSO, respectively). Basal TBARS contents in control Ox-LDL samples in PBS and in DMSO which incubated with $CuSO_4$ were 19.52 ± 3.52 vs. 14.64 ± 3.18 nmol MDA/ mg LDL protein, respectively. There was no significant difference in TBARS contents between control Ox-LDL samples incubated in PBS and in DMSO ($p > 0.05$). Therefore, DMSO was accepted to be used as a diluent in this experiment. In addition, there was no significant difference in the amount of TBARS between groups pre-incubated and no pre-incubated with tested compounds for 30 minutes at 37°C ($p > 0.05$).

TBARS contents in Ox-LDL samples with the presence of various concentrations of γ-OR, ferulic acid, and α-tocopherol, were shown in the unit of nmol MDA/ mg LDL protein (mean ± SEM) (Table 4.4). Percentage of decreased TBARS from individual basal value of Ox-LDL samples (in DMSO, $CuSO_4$ 30 μM, without tested compounds) was also calculated individually before calculation of mean ± SEM (n =10). Compared with ferulic acid and α-tocopherol at the concentration of 10 μM, γ-OR showed a significant decrease in TBARS formation. It was also observed that all of γ-OR, ferulic acid, and α-tocopherol at the concentrations as high as 100 μM and higher, significantly decreased the formation of TBARS substance in the Cu^{2+}-induced LDL oxidation under the condition in this study.

Table 4.4 TBARS contents in Ox-LDL samples during LDL oxidation with various concentrations of γ-OR, ferulic acid, and α-tocopherol

Ox-LDL samples[1]	Pre-incubate 30 min., 37°C		No pre-incubate 30 min., 37°C	
	TBARS Conc.	% Decrease	TBARS Conc.	% Decrease
DMSO (control Ox-LDL)	14.64 ± 3.18 $^{a,c,e,g,l,\dagger,\ddagger,\pi}$	0 $^{a,c,e,g,l,\dagger,\ddagger,\pi}$	14.64 ± 3.18 $^{a,c,e,g,i,\dagger,\ddagger,\pi,\omega}$	0 $^{a,c,e,g,i,\dagger,\ddagger,\pi,\beta}$
γ-OR 1 μM	11.00 ± 2.73 ^{a,†}	18.36 ± 10.07 ^{a,†}	12.76 ± 3.25 ^{a,†,*}	9.05 ± 9.40 ^{a,†}
γ -OR 10 μM	5.04 ± 1.28 ^{c,*}	63.99 ± 10.06 ^{d,*}	5.36 ± 1.46 ^{c,†,*}	63.87 ± 7.37 ^{d,*}
γ -OR 100 μM	3.16 ± 0.96 ^{f,*}	80.21 ± 5.12 ^{f,*}	4.79 ± 1.61 ^{f,*}	65.13 ± 11.01 ^{f,*}
γ -OR 200 μM	3.76 ± 0.91 ^{h,*}	67.73 ± 7.23 ^{h,*}	4.91 ± 1.50 ^{h,*}	62.26 ± 7.19 ^{h,*}
γ -OR 300 μM	2.09 ± 0.58 ^{j,*}	84.96 ± 3.57 ^{j,*}	4.51 ± 1.77 ^{j,*}	70.07 ± 9.40 ^{j,*}
Ferulic acid 1 μM	10.46 ± 2.35 $^{a,\ddagger,\&}$	16.15 ± 14.02 ^{a,‡}	11.21 ± 3.07 $^{a,\ddagger,\&}$	17.05 ± 12.69 ^{a,‡}
Ferulic acid 10 μM	13.83 ± 4.46 ^{c,‡}	8.00 ± 12.26 ^{c,‡}	14.39 ± 4.98 ^{c,‡}	8.84 ± 13.37 ^{c,‡}
Ferulic acid 100 μM	4.25 ± 1.02 $^{f,\ddagger,\&}$	65.39 ± 9.75 $^{f,\&}$	3.75 ± 0.97 $^{f,\ddagger,\&}$	72.71 ± 6.27 $^{f,\&}$
Ferulic acid 200 μM	2.88 ± 0.74 $^{h,\&}$	78.26 ± 5.56 $^{h,\&}$	3.12 ± 0.75 $^{h,\ddagger,\&}$	73.49 ± 9.60 $^{h,\&}$
Ferulic acid 300 μM	1.92 ± 0.50 $^{j,\&}$	86.12 ± 3.47 $^{j,\&}$	2.69 ± 0.64 $^{j,\&}$	77.37 ± 7.15 $^{j,\&}$
α-tocopherol 1 μM	13.28 ± 4.24 $^{a,\pi}$	6.07 ± 16.33 $^{a,\pi}$	13.31 ± 3.82 $^{a,\pi,\omega}$	-4.46 ± 24.60 $^{a,\pi}$
α -tocopherol 10 μM	13.52 ± 5.25 $^{c,\pi}$	17.97 ± 14.54 $^{c,\pi}$	15.77 ± 5.43 $^{c,\pi}$	2.28 ± 24.21 $^{c,\pi,\beta}$
α -tocopherol 100 μM	4.97 ± 1.59 $^{f,\pi}$	67.50 ± 5.64 $^{f,\omega}$	5.97 ± 1.82 $^{e,f,\pi,\omega}$	60.78 ± 9.09 $^{f,\beta,\alpha}$
α -tocopherol 200 μM	2.06 ± 0.39 $^{h,\pi}$	80.06 ± 5.82 $^{h,\omega}$	2.35 ± 0.83 $^{h,\omega}$	84.01 ± 2.70 $^{h,\omega}$
α -tocopherol 300 μM	1.67 ± 0.41 $^{j,\pi}$	85.93 ± 3.92 $^{j,\omega}$	1.77 ± 0.50 $^{j,\omega}$	85.84 ± 4.59 $^{j,\omega}$

Values express mean ±SEM, [1] incubated with $CuSO_4$ 30 μM, 60°C, n =10

$^{\dagger\ddagger\pi*\&\omega\beta}$ *Different symbols indicate significantly different means within each treatment (including control)*

^{abcdefghij} *Different letters indicate significantly different means within each concentration (including control)*

4.5.4 Determination of Relative Electrophoretic Mobility of LDL

Relative electrophoretic mobility (REM) determined the more negative charges of dialyzed LDL particles after the oxidation. It referred to the derivatization of positively charged amino groups in Ox-LDL protein (apo B-100) through the formation of a Schiff base with some reactive aldehydes during oxidation. The agarose gel electrophoresis experiment in this study determined REM of native LDL and Ox-LDL induced by Cu^{2+} with various concentrations of γ-OR, ferulic acid, and α-tocopherol. The REM value was calculated from the migration distance of Ox-LDL to that of native LDL. One sample of agarose gel from the experiment was shown in Figure 4.16. Measured distance from the start point and the REM value of each dialyzed LDL sample were shown in Table 4.5.

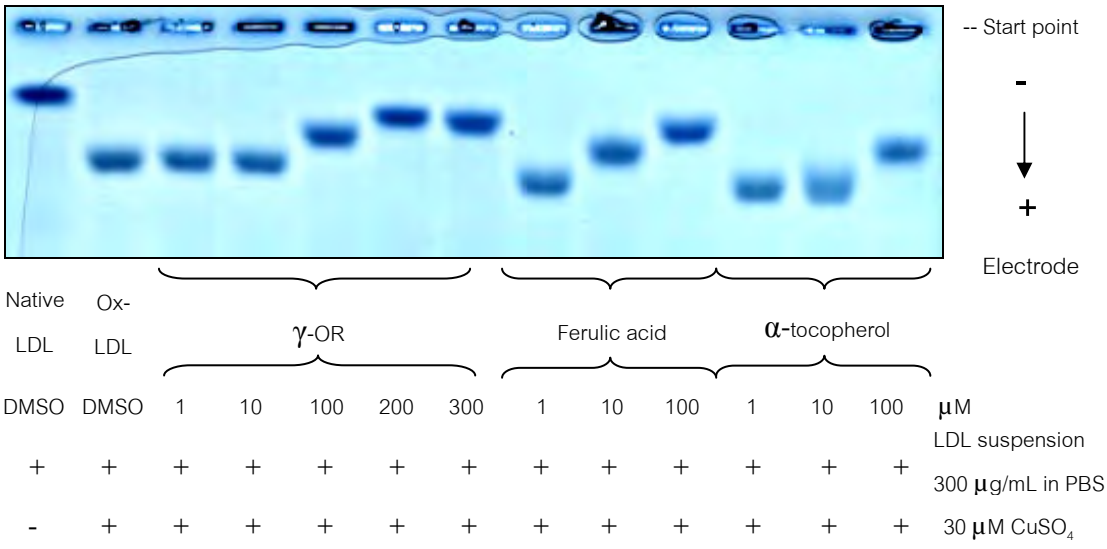


Figure 4.16 REM of LDL sample treated with various concentrations of γ-OR, ferulic acid, and α-tocopherol

In this experiment, the more distance that LDL protein migrated from the start point referred to the more derivatization of positively charged amino groups in apo B-100 of LDL. Native LDL showed the shortest migration from the start point on the agarose gel after electrophoresis, whereas control Ox-LDL migrated longer to positive electric pole (Figure 4.16). The result from this experiment showed that the migration

distance of control Ox-LDL was longer and REM value was higher than those of native LDL with significant (p = 0.004 and 0.038, respectively) (Table 4.5). According to REM values at concentration as high as 100 μM, all tested compounds; γ-OR, ferulic, and α-tocopherol, were able to preserve apo B-100 proteins of LDL from derivatization during oxidation as native LDL vs. control Ox-LDL during the oxidation (Table 4.5).

4.5.5 Determination of Apo B-100 Fragmentation of LDL

Apo B-100 fragmentation of LDL referred to the degree of oxidation of the LDL protein moiety which resulted from the scission of apo B-100. The SDS-PAGE electrophoresis experiment in this study determined the fragmentation of apo B-100 in native LDL and Ox-LDL induced by Cu^{2+} with various concentrations of γ-OR, ferulic acid, and α-tocopherol (Figure 4.17). The values including mean pixel/pixel area, and % raw volume from the stained protein bands, measured by the Gel Documentation (Gel Doc) and image analysis systems (G: Box Chemi SD model) were shown in Table 4.6.

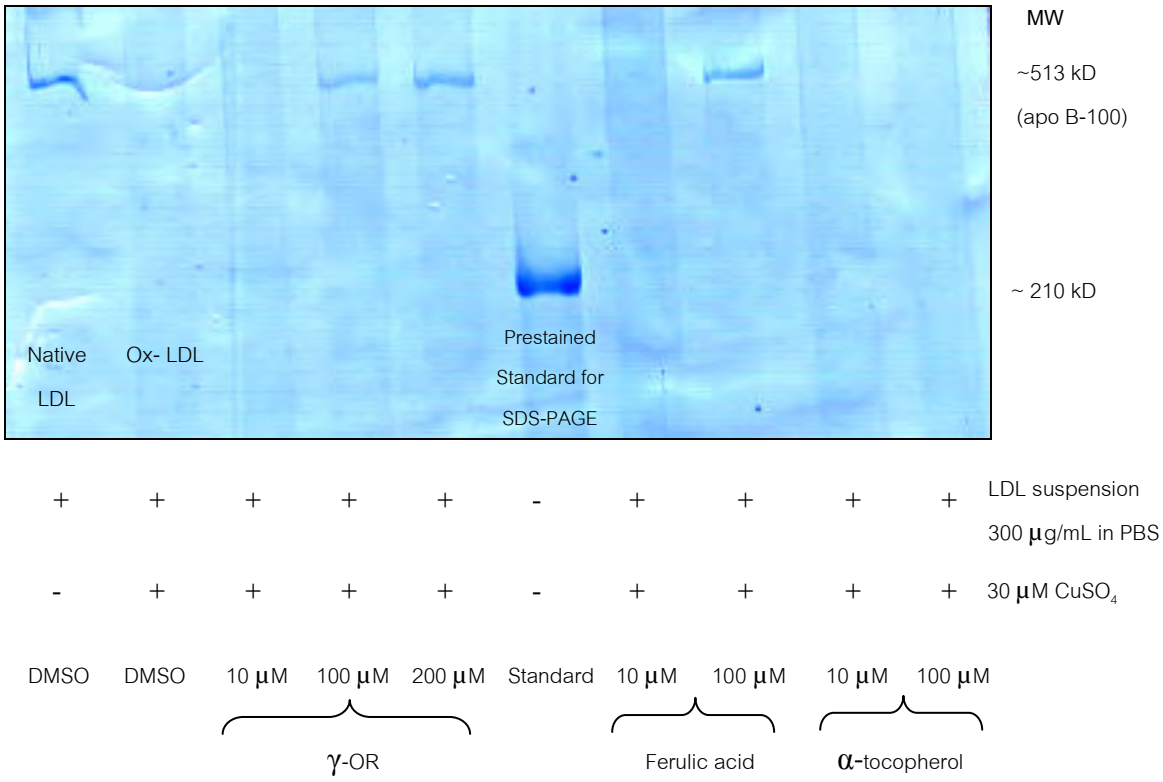


Figure 4.17 One SDS-PAGE gel of LDL treated with various concentrations of γ-OR, ferulic acid, and α-tocopherol in apo B-100 fragmentation

Table 4.5 REM values of LDL samples and distances from the start point in agarose gel electrophoresis after Cu^{2+} induced oxidation with various concentrations of γ-OR, ferulic acid, and α-tocopherol

Ox-LDL samples[1]	Measured distance from the start point (mm)	REM values
Native LDL, DMSO without $CuSO_4$	10.4 ± 0.8 [a, c, e, g, i]	1.00 ± 0.00 [a, c, e, g, i]
Control oxidized LDL, DMSO with $CuSO_4$	16.9 ± 0.9 [b, d, f, h, j]	1.66 ± 0.13 [b, d, f, h, j]
γ-OR 1 μM	16.5 ± 0.9 [b]	1.57 ± 0.15 [a, b]
γ-OR 10 μM	16.3 ± 0.9 [d]	1.62 ± 0.15 [c, d]
γ-OR 100 μM	13.7 ± 0.9 [e]	1.34 ± 0.09 [e, f]
γ-OR 200 μM	11.5 ± 0.6 [g]	1.08 ± 0.07 [g]
γ-OR 300 μM	11.9 ± 0.6 [i]	1.12 ± 0.07 [i]
Ferulic acid 1 μM	18.5 ± 1.0 [b]	1.78 ± 0.19 [b]
Ferulic acid 10 μM	17.1 ± 1.6 [d]	1.70 ± 0.21 [d]
Ferulic acid 100 μM	12.4 ± 0.9 [e]	1.21 ± 0.06 [e]
α-tocopherol 1 μM	18.0 ± 0.8 [b]	1.74 ± 0.20 [b]
α-tocopherol 10 μM	16.9 ± 1.3 [d]	1.68 ± 0.19 [d]
α-tocopherol 100 μM	12.9 ± 0.5 [e]	1.29 ± 0.13 [e]

Values express mean ±SEM, [1] 300 μg protein/mL, incubated with $CuSO_4$ 30 μM, 37°C, n =6

[a b c d e f g h i j] *Different letters indicate significantly different means within each concentration (including control)*

Table 4.6 Values of measured mean pixel/pixel area and % raw volume of LDL samples on the SDS-PAGE gel after Cu^{2+} induced oxidation with various concentrations of γ-OR, ferulic acid, and α-tocopherol

Ox-LDL samples[1]	Mean pixel/ pixel area value	% Raw Volume
Native LDL, DMSO without $CuSO_4$	0.032 ± 0.010 [a, c, e, g]	20.48 ± 5.26 [a, c, e, g]
Control oxidized LDL, DMSO with $CuSO_4$	0.006 ± 0.001 [b, d, f, h]	5.04 ± 0.96 [b, d, f, h]
γ-OR 10 μM	0.009 ± 0.002 [b]	6.69 ± 2.22 [b]
γ-OR 100 μM	0.008 ± 0.001 [d]	6.31 ± 0.60 [d]
γ-OR 200 μM	0.013 ± 0.001 [e]	9.89 ± 0.20 [e]
γ-OR 300 μM	0.016 ± 0.002 [g]	10.99 ± 1.84 [g]
Ferulic acid 10 μM	0.008 ± 0.003 [b]	6.47 ± 2.22 [b]
Ferulic acid 100 μM	0.019 ± 0.002 [c, d]	12.67 ± 0.41 [c]
α-tocopherol 10 μM	0.008 ± 0.001 [b]	5.88 ± 0.91 [b]
α-tocopherol 100 μM	0.014 ± 0.002 [c, d]	12.25 ± 2.12 [c, d]

Values express mean ±SEM, [1] 300 μg protein/mL, incubated with $CuSO_4$ 30 μM, 37°C, n = 3

[a b c d e f g h] *Different letters indicate significantly different means within each concentration (including control)*

After electrophoresis it was observed that native LDL had the highest density of band while control Ox-LDL had the lowest density of band with small fragments moving along to the end on a SDS-PAGE gel (Figure 4.17). Mean pixel/pixel areas and % raw volumes demonstrated the degree of apo B-10 fragmentation during Cu^{2+}-induced LDL oxidation (Table 4.6). The results confirmed that native LDL had the lowest degree of apo B-10 fragmentation while control Ox-LDL had the highest degree of apo B-100

fragmentation. Morover mean pixel/pixel areas and % raw volumes of control Ox-LDL band were markedly less than those of native LDL (p = 0.022 and 0.020, respectively).

Comparing to control Ox-LDL, ferulic acid and α-tocopherol at 100 μM inhibited apo B-100 fragmentation of Ox-LDL during the oxidation ($p < 0.05$) while γ-OR at the same concentration did not show the inhibitory effect ($p > 0.05$). However, at the higher concentrations of 200 and 300 μM, γ-OR showed significantly more % raw volumes than those of control Ox-LDL ($p < 0.05$) (Table 4.6). This study indicated that it needed γ-OR at the concentration as high as 200 μM or more to inhibit apo B-100 fragmentation of Ox-LDL in this system.

CHAPTER V

DISCUSSION

It has been shown in many animal and human studies that γ-OR could lower blood cholesterol (10, 11, 13, 14, 68-77). However, the mechanism for this activity has been still unclear. Because of the similar chemical structure to cholesterol, γ-OR was believed that it could inhibit cholesterol absorption due to inhibition of micellar solubilization of cholesterol. The proposed mechanism of γ-OR on its hypocholesterolemic activity has attracted the main intention on micellarization step during digestion and absorption into intestinal cells, enterocytes. Both synthetic micelle experiment and simulated digestion method which mimicked the body digestive system were conducted in this study to answer this hypothesis. However, the cholesterol-lowering mechanism in blood system should not be concerned only cholesterol absorption but also cholesterol biosynthesis. Mevalonate, a key intermediate in cholesterol biosynthesis, is formed by HMG-CoA reductase (48). On the other hand, inhibition of HMG-CoA reductase activity can reduce endogeneous cholesterol biosynthesis. Therefore, the effect of γ-OR on cholesterol biosynthesis was also determined in this study by evaluated the inhibitory activity of γ-OR against HMG-CoA reductase.

To prevent the interference, this study used polished Jasmine white rice containing no rice bran for conducting simulated digestion. Vehicle oil was used for two reasons; to solubilize γ-OR due to the less solubility of γ-OR in aqueous solution and to mimic edible oil as an ingredient in food meal. Some components of γ-OR, mainly sitostanyl ferulate and campestanyl ferulate, and lesser amounts of sitosteryl ferulate and campesteryl ferulate, can be also found mostly in the inner pericarp of corn, wheat, rye, and triticale grains (78). Therefore, peanut oil was used as vehicle oil for γ-OR and cholesterol infusion as a result of its fatty acid profile close to that of rice bran oil with the absence of γ-OR. The evidence in Figure 4.3 also confirmed that there was no γ-OR in rice meal mixed with vehicle peanut oil used as food sample in this study. In general,

edible peanut oil contains palmitic acid (C16:0) 11.6 %, stearic acid (C18:0) 3.1%, oleic acid (C18:1) 46.5%, linoleic acid (C18:2) 31.4%, and other minor fatty acids (79), while rice bran oil is composed of palmitic acid 16.4 %, stearic acid 2.1%, oleic acid 43.8%, linoleic acid 34.0% and other minor fatty acids. The fatty acid composition on the labeled nutrition fact of peanut oil used in this study was similar to the data mentioned above. When considering amount of vitamin E, antioxidant presented approximately 20% of the oil, it did not affect the simulated digestion experiments in Phase II and Phase III which determined the incorporation of cholesterol into micelles not antioxidant activity. All tests also used the same peanut oil as vehicle oil to prevent the interference.

5.1 Bioaccessibility and Bioavailability of γ-OR

5.1.1 Bioaccessibility of γ-OR Using Simulated Digestion Method

In this *in vitro* study, γ-OR showed its good bioaccessibility, but poor bioavailabilty. Based on the results from simulated digestion, when there was only γ-OR or cholesterol alone in the system, it showed that approximately 50% of total γ-OR incorporated into mixed micelles, similar to cholesterol (mean ± SEM; 53.7 ± 3.6% vs. 56.3 ± 1.3%, respectively). In addition, all ten of γ-OR components incorporated into mixed micelles with similar efficiency (Figure 4.4), and exceeded 80% of recoveries after the oral, gastric, and small intestinal phases of simulated digestion. It suggested that γ-OR incorporated into mixed micelles as intact form and was not much degraded by digestive enzymes in the oral, gastric, and small intestinal phases. This study could not confirm whether and how much γ-OR cleaved to be ferulic acid and sterols during simulated digestion. Ferulic acid and sterols in chyme and micelle fraction were not determined to solve the assumption due to some experimental limitation. However, some studies reported that γ-OR could be hydrolyzed by some digestive enzymes (28, 37, 80). Pancreatic cholesterol esterase catalyzed a sterol-specific reaction with some components of γ-OR; campesteryl ferulate and sitosteryl ferulate, and produced sitosterol and campesterol and ferulic acid; whereas cycloartenyl ferulate and 24-methylenecycloartanyl ferulate remained unhydrolyzed (28, 37). Moreover, sitostanyl ferulate was hydrolyzed by cholesterol esterase (84.7 ± 3.8%) and pancreatin (47.3 ±

8.3%) after 4-hour incubation. Whereas γ-OR was hydrolyzed only by cholesterol esterase (56.3 ± 7.3%) after 4-hour incubation but not by pancreatin (80). Nonetheless, lipase and phospholipase A_2 had no efficiency to hydrolyze γ-OR (28, 37). Although pepsin and low pH degraded γ-OR, it was not conclusive whether the ester bond of γ-OR was breakdown by pepsin (28).

5.1.2 Bioavailability of γ-OR

Phytosterols have been considered to be nonabsorbable, however a small but definite absorption occurred (81). The absorption at 0.6 - 7.5% of the administered amount was reported in several studies using radioactive sitosterol in human (82-84), however percentage of absorption varied inversely with the dose (83). One study reported lower values for % absorption of sitosterol (0.51%), campesterol (1.9%), campestanol (0.16%), and sitosterol (0.04%) (81). In general, the mean fractional absorption of campesterol, sitosterol, campestanol, and sitostanol were 10%, 5%, 2%, 1%, respectively, with a marked variation interindividually and among different studies (85). Therefore, absorption of γ-OR, a mixture of phytosterols esters, was also predicted to be low. In an *in vitro* study, the uptake of intact γ-OR by C2BBe1 cells (ATCC CRL-2102) was not detected after 2-hour incubation of γ-OR synthetic micelles (28). Nevertheless, the uptake of intact γ-OR by Caco-2 intestinal cells was detected in this present study. Percentage of γ-OR absorption by Caco-2 cells after 4-hour incubation was 2.46 ± 0.16 % (mean ± SEM, per mg of cells protein) of total γ-OR in micellarized γ-OR after simulated digestion. Differences on incubation time period (2-hour vs. 4-hour), type of cell line (C2BBe1 vs. Caco-2), sizes of monolayer surface areas, amounts of cells (96-well plates vs. T-75 flasks), and micelle preparation (synthetic vs. natural micelles) may affect the ability to detect accumulated γ-OR in cells.

5.2 Effect of γ-OR on the Efficiency of Cholesterol Micellarization

To evaluate the inhibitory effect of γ-OR on the efficiency of cholesterol micellarization, synthetic micelles and simulated digestion experiments that contained cholesterol with the absence or presence of γ-OR were measured. The result from the

synthetic micelle experiment in this study did not clearly show that γ-OR could decrease the efficiency of cholesterol micellarization in small dose of γ-OR usage. At the molar ratio of γ-OR to cholesterol higher than 15 fold- (when cholesterol concentration was less than 100 μM and γ-OR was 1500 μM), a decrease in the efficiency of cholesterol micellarization was observed (Figure 4.8). However, at cholesterol concentration of 400 μM, the efficiency of cholesterol significantly decreased without the effect from γ-OR. It may be possible that high level of cholesterol can decrease its own efficiency to incorporate into micelles during digestion. On the other hand, it may explain that cholesterol has its own threshold of the incorporation into mixed micelles and the efficiency of γ-OR on inhibiting cholesterol micellarization is also limited. In addition, it may suggest that γ-OR, especially at low concentration and less than 2-fold molar ratio to cholesterol, cannot decrease the efficiency of cholesterol micellarization when high concentration of cholesterol presents. In other words, a large amount of cholesterol might interfere competitively with the micellarization of γ-OR in the intestinal lumen.

The effect of γ-OR on the efficiency of cholesterol micellarization was also evaluated using simulated digestion method. No inhibitory effect of γ-OR on the efficiency of cholesterol micellarization was observed. The 10- and 20-fold molar ratio of γ-OR to cholesterol (1.3 μM cholesterol) did not decrease the efficiency of cholesterol incorporation into mixed micelles during simulated digestion. The concentrations of cholesterol used in this simulated digestion condition was lower than normal daily cholesterol intake because of some limitation of procedure. However, it suggested that γ-OR at very low concentration (13 and 26 μM) had no inhibitory effect on cholesterol micellarization although it was high molar ratio to cholesterol (10- and 20-fold). The evidence that found in this study may be explained by the chemical structure of γ-OR, the esterified form with ferulic acid, less polar than free sterols and cholesterol. In addition, only some components of γ-OR can be hydrolyzed by pancreatic cholesterol esterase. Major components of γ-OR including cycloartenyl ferulate and 24-methylenecycloartanyl ferulate accounting ~70% of total component are unhydrolyzed. Therefore, γ-OR has limitation to compete with free cholesterol for incorporation into micelles in the same space and condition as cholesterol. Unless the molar ratio to

cholesterol of γ-OR concentration is very high, γ-OR may be able to take place the space of cholesterol in micelles.

Approximate daily volume of digestive juice is 7.5 L (86). If the concentrations of γ-OR and cholesterol in the synthetic micelles experiment be converted from μM to mg, assuming that body digestive fluid in an adult accounts about 2 L per one meal, it means that an adult requires to consume at least 1800 mg of γ-OR (1500 μM) for less than 80 mg of cholesterol intake (100 μM) to expect a decrease in the efficiency of cholesterol micellarization during digestion. Nevertheless, 1800 mg of γ-OR (1500 μM) cannot affect the efficiency of cholesterol micellarization if dietary cholesterol consumption is more than 300 mg per day (400 μM). To expect this inhibitory effect of γ-OR on cholesterol micellarization, more than 450 g of high γ-OR rice bran oil (4 g γ-OR / kg of oil) need to be consumed. In case of rice consumption to reach 1800 mg of γ-OR, more than 4,316 g of brown rice grain [41.7 mg γ-OR /100 g of dried rice grain (87)] or 2,467 g of purple rice grain-Kumdoisaket genotype [72.95 mg γ-OR/100 g of dried grain (88)] are needed. Therefore, consuming cooked purple rice with high γ-OR rice bran oil may not reach the high amount mentioned above. Supplement with γ-OR extract may be an alternative way to obtain more amount of of γ-OR.

5.3 Effect of γ-OR on the Uptake of Cholesterol by Caco-2 cells

Many studies indicated that about 50% of dietary cholesterol was absorbed in normal subjects during the test meal. However, % cholesterol absorption varied from 25 to 80 among different individuals or different races (89). At high sterol concentrations, competition of sitosterol with cholesterol for uptake into intestinal epithelium cells could be observed (20). In the present *in vitro* study, cholesterol was absorbed into Caco-2 cells for approximately 8 % of micellarized cholesterol per mg of cell protein. Such a low percentage of cholesterol absorption observed may occur because of very small amount of cholesterol (1.3 μM) used during simulated digestion. However, the efficiency of γ-OR on inhibition of cholesterol absorption was detected (Figure 4.8). Although at low concentration of γ-OR (less than 500 μM) in synthetic micelles experiment did not show inhibitory effect on the efficiency of cholesterol micellarization,

low concentration of γ-OR (26 μM) but high molar ratio to cholesterol (20-fold) showed the inhibition activity on cholesterol uptake by intestinal cells (Figure 4.11 and 4.12). In a study, synthetic micelles with γ-OR at molar ratio 1:1 to cholesterol (100 μM) did not show effect on decreasing cholesterol uptake by C2BBe1 cells after 2-hour incubation (28). It suggested that γ-OR higher than 10-fold molar ratio to cholesterol exerted the inhibitory effect on the absorption of cholesterol although the inhibition of cholesterol micellarization did not succeed.

Plant sterols (1.2-2.2 g of sterols/ day), sterol esters (1.5 – 3.3 g of sterols/ day), and stanol esters (~3 g of sterols/ day) were effective to lower serum cholesterol in humans (85). In those studies, sitosterol was a mainly interest compound. There were several randomized clinical trials in humans that showed lower serum total cholesterol and LDL-C after 300 mg of γ-OR administration daily for 8-13 weeks in postmenopausal women (69, 90) and in hypercholesterolemic subjects (77, 91). However, no effect on metabolic parameters was observed after 500 mg of γ-OR administration daily for 9 weeks in healthy young men (92). The results from this *in vitro* study and the effective dose of γ-OR in clinical studies really suggest that hypocholesterolemic activity of γ-OR reported in animal and human studies is not likely due to inhibition of cholesterol micellarization. In addition, although γ-OR did not succeed in competing with free cholesterol during micellarization, it may reduce the cholesterol absorption into enterocytes by interfering cholesterol esterase activity and reduce the hydrolysis of cholesteryl esters (28). The ester form of cholesterol could not be absorbed by enterocytes without prior hydrolysis (43).

Furthermore, it is possible that γ-OR may inhibit the absorption of micellarized cholesterol into enterocytes. Gamma-oryzanol may affect one or more transporter proteins of enterocytes regulating cholesterol absorption. Many intestinal transporter proteins and enzymes involved in cholesterol and other sterols absorption such as Niemann-Pick C1 Like1 (NPC1L1), ATP- binding cassette transporter G5 (ABCG5), ATP-binding cassette transporter G8 (ABCG8), ATP-binding cassette transporter A1 (ABCA1), and Acyl CoA: cholesterol acytransferase type 2 (ACAT2). The mechanism for cholesterol and phytosterol absorption in the intestine was complicate and partially

known. First, cholesterol and phytosterols were absorbed into enterocytes by NPC1L1 transporter protein localizing on the surface of the enterocytes (50, 93). Second, absorption of cholesterol and phytosterols was limited by ABCG5 and ABCG8 transporter proteins localizing on the apical surface of enterocytes. ABCG5 and ABCG8 transporters possibly discriminating structural differences in various plant sterols worked as heterodimer in the endoplasmic reticulum and secreted the absorbed cholesterol and phytosterols from enterocytes back into the lumen of intestine (94). In hepatocytes, ABCG5 and ABCG8 secreted cholesterol into bile, the major pathway for cholesterol elimination in mammals. Third, ACAT2 enzyme preferred to esterify intracellular cholesterol rather than sitosterol in order to form and excrete chylomicrons into the circulation. Thus it effectively eliminated phytosterols from the absorption process (95). Forth, sterols stimulated ABCA1 expression to pump free cholesterol back to the lumen of intestine (96). Nevertheless, the effect of γ-OR on the regulation of those transporter proteins did not conduct in this present study. Further study focusing on the regulation of those transporter proteins is needed to understand more in the mechanism and interaction of γ-OR on cholesterol absorption within the enterocytes.

5.4 Effect of γ-OR and Ferulic Acid on the HMG-CoA Reductase Activity

In addition to the inhibition of cholesterol absorption, hypocholesterolemic activity of γ-OR may also due to other mechanisms such as the inhibition of cholesterol biosynthesis. The inhibition of HMG-CoA reductase, a key enzyme for endogenous cholesterol synthesis, was generally considered to reduce hepatic cholesterol content and thus caused the up-regulation of hepatic LDL receptors resulting in enhanced LDL uptake and a reduction in serum cholesrerol (97). Statins, the HMG-CoA reductase inhibitors, were widely used in clinical practice to lowering serum cholesterol level in hypercholesterolemic patients. Phytosterols may affect cholesterol synthesis, as β-sitosterol has been shown to inhibit HMG-CoA reductase gene expression in Caco-2 cells (98).

To evaluate the inhibitory effect of γ-OR on cholesterol biosynthesis, its inhibitory activity against HMG-CoA reductase was measured in this study. Ferulic acid has also

been interested in the HMG-CoA reductase activity assay and antioxidation experiments. It was the important part in γ-OR structure cleaved by pancreatic cholesterol esterase during digestion. The result showed that γ-OR was a HMG-CoA inhibitor. The IC_{50} values of simvastatin, γ-OR, and ferulic acid were not detected but at the same concentration, γ-OR and ferulic acid tended to be more potent inhibitors against HMG-CoA reductase activity than that of simvastatin. In addition, ferulic acid was likely the most potent HMG-CoA reductase inhibitor than γ-OR and simvastatin, respectively.

A study in rats has been shown that γ-OR could activate the hepatic LDL receptor and increase HMG-CoA reductase mRNA and fecal neutral sterol and bile acid excretion (99). It may explain that mechanism of hypocholesterolemic activity of γ-OR involved the inhibition of cholesterol absorption in the intestine and HMG-CoA reductase activity for cholesterol synthesis, and the increased catabolism of cholesterol resulted from the reduction in cholesterol reabsorption in the intestine. Lowering plasma and liver cholesterol levels in rats fed diet containing γ-OR may be due to the increased hepatic LDL-receptor expression which facilitated the lowering of LDL-C and increase LDL-C uptake in liver, the increased cholesterol 7α-hydroxylase (CYP7A1) expression which facilitated cholesterol catabolism to synthesize bile acid and excrete fecal neutral sterols, and then the upregulation of HMG-CoA reductase expression to synthesize cholesterol for the cholesterol homeostasis *in vivo* (99).

5.5 Antioxidant Activities of γ-OR against LDL Oxidation

Prevention of the Ox-LDL formation by antioxidants is proposed to be helpful in decreasing the risk of cardiovascular diseases. Several *in vitro* studies reported the antioxidant activities of γ-OR major components (6-8). Therefore, the antioxidant activity of γ-OR against LDL oxidation was also investigated in this study. Although γ-OR was poorly absorbed into enterocytes, some amounts of unabsorbed γ-OR may be able to transport to the liver via portal vein system. In the part of absorbed γ-OR, it is possibly transported in the chylomicron and be taken up by liver in the form of chylomicron remnant, then be secreted and circulate in blood stream with lipoproteins as a lipid

constituent of VLDL particles. Thus, γ-OR is supposed to play the role as antioxidant for lipoprotein pretection, especially LDL, from oxidation during transportation along to peripheral tissues.

In part of ferulic acid, it was suggested that ferulic acid could be absorbed in intestine by passive diffusion, a facilitated transport mechanism, or a Na^{2+}/dependent carrier-mediated transport mechanism. It was conjugated by intestincal conjugation enzyme and then all absorbed ferulic acid as conjugated forms could be secreted into mesenteric vein and transport to peripheral tissues (100). Furthermore, free ferulic acid could be absorbed into gastric mucosa cells, transported into the portal vein and the liver, and conjugated as mentioned above. The remaining free ferulic acid and its conjugates entered blood circulation (101). Many studies reported that ferulic acid played a role as an antioxidant inhibiting lipid peroxidation (102), and LDL oxidation (103), and scavenging oxgen radicals (104).

The antioxidant activity with varied concentrations of γ-OR against LDL oxidation was investigated and compared with ferulic acid and α-tocopherol. A dialyzed LDL sample of individual healthy volunteer was used for each susceptibility test to LDL oxidation. The results showed that γ-OR exhibited weaker antioxidant activity than ferulic acid and α-tocopherol in propose to prolong the time to generate lipid peroxidation. At the concentration of 50 μM, γ-OR prolonged the lag time and the decomposition time of conjugated dienes formation. Also it decreased the propagation rate and the maximal production of conjugated dienes during Cu^{2+}-induced LDL oxidation. Whereas, lower concentrations of ferulic acid and α-tocopherol (10 μM and 5 μM, respectively) were able to prolong the lag time of conjugated dienes formation. However, γ-OR, ferulic acid, and α-tocopherol prolonged the decomposition time and decreased the propagation rate and the maximal production of conjugated dienes only at their concentrations of 50 μM. The earlier studies demonstrated that γ-OR possed a dose-dependent DPPH scavenging activity, and AMVN initiated lipid peroxidation but the acitivity of γ-OR < ferulic acid < α-tocopherol (30, 105). In addition, γ-OR was able to inhibit lipid soluble organic radicals at concentration of 50-100 μM (30), which was correlated to the results in the present study.

Based on the results of TBARS, there was no significant difference in the amounts of TBARS between groups pre-incubated and no pre-incubated with tested compounds for 30 minutes at 37°C. It suggested that each compound at effective concentration was able to decrease the decomposition of LDL to form aldehyde substances during the oxidation even without pre-incubation before exposed to free radicals. Nevertheless, γ-OR at the concentration of 10 μM decreased TBARS formation after *in vitro* oxidation, which was stronger than the activity of ferulic acid, and α-tocopherol. This finding was correlated to the earlier study demonstrating the inhibition effect of ferulic acid on the hydroperoxides formation with almost the same as that of α-tocopherol. Moreover, ferulic esters such as cycloartenyl ferulate, 24-methylene-cycloartanyl ferulate, and γ-OR could inhibit hydroperoxide formation more strongly than ferulic acid did (105).

Inhibition effect on the alteration of the surface charge on apo B-100 by REM determination indicated that at the same concentration as high as 100 μM in all compounds were able to protect apo B-100 protein of LDL. In order to inhibit apo B-100 fragmentation of Ox-LDL, γ-OR at the concentration as high as 200 μM or more was required. However, only 100 μM of ferulic and α-tocopherol were needed for the same activity. These results may suggest that γ-OR exhibited weaker antioxidant activity than ferulic acid and α-tocopherol in propose to inhibit the derivertization of apo B-100 protein in LDL during oxidation. The mechanisms of antioxidant action and antioxidative sensitivity of γ-OR, ferulic acid and α-tocopherol may be different and need more investigation.

CHAPTER VI

CONCLUSION

The results from this study showed that γ-OR had good bioaccessibility. It incorporated efficiently into mixed micelles during simulated digestion, similar to cholesterol. Nevertheless, γ-OR had poor bioavailability to be absorbed into intestinal cells. Also γ-OR at low concentration or low molar ratio to cholesterol did not decrease the efficiency of cholesterol on incorporation into micelles. Synthetic micelles experiment indicated that high concentrations of γ-OR at 1500 μM did not significantly decrease the efficiency of cholesterol micellarization when cholesterol concentration was as high as 400 μM. In addition, 10- and 20-fold molar ratios of γ-OR to cholesterol did not inhibit the efficiency of cholesterol micellarization during simulated digestion when γ-OR concentration was very low (13 and 26 μM, respectively). Nevertheless, very low concentration (26 μM) of γ-OR but high molar ratio to cholesterol (20-fold) significantly decreased cholesterol uptake by Caco-2 intestinal cells. In addition to the inhibition effect on cholesterol absorption, γ-OR showed the inhibitory effect on the HMG-CoA reductase activity which was not less than that of simvastatin, a well known HMG-CoA reductase inhibitor. Ferulic acid cleaved from some components of γ-OR by cholesterol esterase during digestion also exhibited the inhibitory effect on HMG-CoA reductase activity. These findings suggest that the hypocholesterolemic activity of γ-OR found in earlier human and animal studies is possibly due to the inhibition of cholesterol biosynthesis, rather than the inhibition of cholesterol micellarization.

The susceptibility tests of LDL to *in vitro* oxidation monitoring conjugated dienes production showed that γ-OR at concentration of 50 μM prolonged the lag time and the decomposition time of conjugated dienes formation, and also decreased the propagation rate and the maximal production of conjugated dienes during Cu^{2+}-induced LDL oxidation. Whereas lower concentrations of ferulic acid and α-tocopherol (10 μM and 5 μM, respectively) were able to prolong the lag time of conjugated dienes formation. However, γ-OR, ferulic acid, and α-tocopherol prolonged the decomposition

time and decreased the propagation rate and the maximal production of conjugated dienes only at concentration of 50 μM. In other words, γ-OR exhibited weaker antioxidant activity than those of ferulic acid and α-tocopherol in propose to prolong the time to generate lipid peroxidation. To inhibit the formation of secondary intermediates of lipid peroxidation, γ-OR at the concentration of 10 μM decreased TBARS formation after *in vitro* oxidation, which was stronger than the activities of ferulic acid, and α-tocopherol. Moreover, it was found that at the same concentration as high as 100 μM, all of γ-OR, ferulic and α-tocopherol were able to protect apo B-100 protein of LDL from alteration of the surface charge during oxidation to be as same as the native LDL. However, in order to inhibit apo B-100 fragmentation of Ox-LDL, γ-OR at the concentrations as high as 200 μM or more was needed whereas only 100 μM of ferulic and α-tocopherol were required for the same activity. These results suggested that γ-OR exhibited weaker antioxidant activity than those of ferulic acid and α-tocopherol in propose to inhibit the derivertization of apo B-100 protein in LDL during oxidation. In conclusion, in this *in vitro* study, γ-OR showed weaker antioxidant activity against LDL oxidation than ferulic acid and α-tocopherol to inhibit the primary intermediates of lipid peroxidation and the derivertization of apo B-100 protein, but it was stronger than those compounds to inhibit the secondary intermediates of lipid peroxidation. Therefore, it may also suggest that the mechanisms of antioxidant action and antioxidative sensitivity of γ-OR, ferulic acid and α-tocopherol were different and need more investigation.

SUGGESTIONS FOR FURTHER STUDIES

Further studies in nutrigenomic approach are needed to understand whole mechanism of hypocholesterolemic activity of γ-OR such as gene expression on the regulation of receptors, transporter proteins in enterocytes, and effects of enzymes activities regulated in cholesterol absorption. In addition, more experiments are also needed to conduct to understand the mode of action of γ-OR in antioxidative activities. Moreover, in order to gain the benefit of lowering blood cholesterol and antioxidant effects of γ-OR in small intestine, research and development on solubility and bioavailability of γ-OR in forms of food or supplements should be pursued.

THE BENEFITS OF THIS STUDY AND RECOMMENDATION:

Results from this study can be useful for nutritionist, food sciencetists, food technologists, scientists, pharmacists, and scientific researchers to understand the bioavailability, some part of the mechanism of hypocholesterolemic activity of γ-OR, and the antioxidant potential of γ-OR on LDL oxidation. Food sciencetists and food technologists should further develop γ-OR in the food having high solubility and bioavailability for consuming in small dose usage. Then, it can consequently lead to an added value of rice bran for nutraceutical approach as functional food and health promotion benefit for consumers worldwide.

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APPENDICES

APPENDIX A: Certificate of Approval from the Ethical Review Committee

บันทึกข้อความ

ส่วนงาน คณะกรรมการพิจารณาจริยธรรมการวิจัยในคนฯ วิทยาลัยวิทยาศาสตร์สาธารณสุข โทร.88147

ที่ จว 951/2551 **วันที่** 9 กรกฎาคม 2551

เรื่อง แจ้งผลพิจารณาจริยธรรมการวิจัย

เรียน คณบดีคณะสหเวชศาสตร์

สิ่งที่ส่งมาด้วย 1. ใบรับรองผลการพิจารณา
2. ข้อมูลสำหรับประชากรตัวอย่างหรือผู้มีส่วนร่วมในการวิจัย
3. ใบยินยอมของประชากรตัวอย่างหรือผู้มีส่วนร่วมในการวิจัย

ตามที่ นางสาวกิตฉา จันทร์ดา นิสิตระดับศุษฎีบัณฑิต สหสาขาวิชาชีวเวชศาสตร์ บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย ได้เสนอแก้ไขโครงการวิจัยที่ 05202/51 เรื่อง การดูดซึมและการนำ γ - oryzanol ไปใช้โดย Caco - 2 cells และฤทธิ์ต้านการเกิดอนุมูลอิสระในไลโปโปรตีนชนิดความหนาแน่นต่ำ (BIOAVAILABILITY OF γ - ORYZANOL USING CACO - 2 CELLS CULTURE MODEL AND ITS ANTIOXIDANT ACTIVITIES AGAINST LOW - DENSITY LIPOPROTEIN OXIDATION) เพื่อให้คณะกรรมการพิจารณาจริยธรรมการวิจัยในคน กลุ่มสหสถาบัน ชุดที่ 1 จุฬาลงกรณ์มหาวิทยาลัย พิจารณาจริยธรรมการวิจัยความละเอียดแจ้งแล้วนั้น

การนี้ คณะกรรมการฯ ได้พิจารณาแล้ว เมื่อวันศุกร์ที่ 13 มิถุนายน 2551 มีมติให้ผ่านการพิจารณาจริยธรรมการวิจัยได้

จึงเรียนมาเพื่อโปรดทราบ

(ผู้ช่วยศาสตราจารย์ ดร.นันทรี ชัยชนะวงศาโรจน์)
กรรมการและเลขานุการ
คณะกรรมการพิจารณาจริยธรรมการวิจัยในคน
กลุ่มสหสถาบัน ชุดที่ 1 จุฬาลงกรณ์มหาวิทยาลัย

The Ethical Review Committee for Research Involving Human Research Subjects, Health Science Group, Chulalongkorn University
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Institute Building 2, 4 Floor, Soi Chulalongkorn 62, Phyat hai Rd., Bangkok 10330, Thailand,
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COA No. 066/2008

Certificate of Approval

Study Title No.05202/51.... : **BIOAVAILABILITY OF γ - ORYZANOL USING CACO - 2 CELLS CULTURE MODEL AND ITS ANTIOXIDANT ACTIVITIES AGAINST LOW - DENSITY LIPOPROTEIN OXIDATION**

Principle Investigator : Miss Kittana Chanda, Doctor of Philosophy Program in Biomedical Sciences Graduate School

Place of Proposed Study/Institution : Faculty of Allied Health Sciences, Chulalongkorn University

The Ethical Review Committee for Research Involving Human Research Subjects, Health Science Group, Chulalongkorn University, Thailand, has approved constituted in accordance with the International Conference on Harmonization – Good Clinical Practice (ICH-GCP) and/or Code of Conduct in Animal Use of NRCT version 2000.

Signature: ..... Signature: .....

(Associate Professor Prida Tasanapradit, M.D.) Chairman

(Assistant Professor Dr. Nuntaree Chaichanawongsaroj) Secretary

Date of Approval : 10 June 2008 Approval Expire date : 9 June 2009

The approved investigator must comply with the following conditions:

1. The research/project activities must end on the approval expired date of the Ethical Review Committee for Research Involving Human Research Subjects, Health Science Group, Chulalongkorn University (ECCU). In case the research/project is unable to complete within that date, the project extension can be applied one month prior to the ECCU approval expired date.
2. Strictly conduct the research/project activities as written in the proposal.
3. Using only the documents that bearing the ECCU's seal of approval with the subjects/volunteers (including subject information sheet, consent form, invitation letter for project/research participation (if available); and return the first subject's copy of the above documents to the ECCU.
4. Report to the ECCU for any serious adverse events within 5 working days
5. Report to the ECCU for any change of the research/project activities prior to conduct the activities.
6. Abstract submission (in a CD ROM format) is required for a one year (or less) research/project (the submission should be within 30 day after the completion of the research/project)
7. Annual progress report (in a CD ROM format) is needed for a two- year (or more) research/project (the submission should be within 60 day after the completion of the research/project)

APPENDIX B: Potassium Bromide Solution Preparation for LDL Isolation

Potassium bromide (KBr) solution for LDL isolation can be prepared according to the formula below.

1. Prepare Sodium azide solution at concentration 10% w/v in distilled water
2. Prepare Sodium EDTA solution at concentration 10% w/v in distilled water
3. Dissolve solid KBr with appropriate volume of distilled water, mix with sodium azide and sodium EDTA solution at the proportion as shown in the table for 1 L of KBr solution or indicated amounts of those for other final volumes of KBr solution according to this formula
4. Make up the volume with distilled, de-ionized water to 1 L of final solution (for 1 L of KBr solution preparation) or to other indicated final volume of KBr solution based on this formula

Density of KBr Solution	Chemical materials needed for 1 L of KBr Solution
d = 1.006	8.235 g solid KBr + 1 mL Sodium azide solution + 1 mL Sodium EDTA solution
d = 1.019	29.924 g solid KBr + 1 mL Sodium azide solution + 1 mL Sodium EDTA solution
d = 1.063	89.275 g solid KBr + 1 mL Sodium azide solution + 1 mL Sodium EDTA solution
d = 1.210	326.188 g solid KBr + 1 mL Sodium azide solution + 1 mL Sodium EDTA solution

5. Check the density of KBr solution by weighing 100 μL of solution with analytical balance (4 decimals of mg unit) for 10 times. (Example: 100 μL of density 1.019 KBr solution should weight 0.1019 mg)
6. Store the solution at 4°C

APPENDIX C: Preparation of Phosphate Buffer Saline, pH 7.4 Solution for LDL Dialysis and LDL Oxidation Experiments

Phosphate buffer saline (PBS) solution, pH 7.4, containing 0.01 M phosphate, 0.0027 M KCl, and 0.138 M NaCl, can be prepared according to the formula below.

1. Preparation for 1 L of 10X 0.01 M phosphate buffer (or 0.1 M of stock phosphate buffer) can be conducted by combine following chemical materials in 900 mL of distilled water

$Na_2HPO_4 \cdot 2H_2O$	8.2 mM	=	14.595 g
$NaH_2PO_4 \cdot H_2O$	1.8 mM	=	2.483 g
KCl	0.027 M	=	2.013 g
NaCl	1.38 M	=	80.647 g

2. Adjust pH of solution above to be 7.4 using 1N HCl if needed, and add up distilled water to final volume 1 L of 10X stock solution

3. Dilute 1:10 with distilled water before use in the experiment (i.e. 100 mL of stock solution + 900 mL distilled water to make up 1 L of 0.01 M PBS that contained 0.0027 M KCl, and 0.138 M NaCl)

4. Store the buffer solution at 4˚C

APPENDIX D: BCA (Pierce) Protein Assay

Protein contents of cell suspension were determined using BCA protein assay (Pierce, Thermo Fisher Scientific, Inc.) as following procedure.

1. Calculate the amount of working reagent required for the analysis in triplicate (prepare about at least 2 mL extra; use 0.2 mL of working reagent for 1 test or 1 well of 96-well microtiter plate)

Blank (n=1)

Standards (n= X; Example: X = 6 standards)

Samples (n= Y; Example: Y = 18 samples)

Total wells needed = 3(1+X+Y) = Z wells on 96-well flat bottom plate

→ 3(1+6+18) =75

Total volume of working reagent needed = (0.2 mL*Z)+2 mL

→ (0.2*75)+2 = 17 → 20 mL

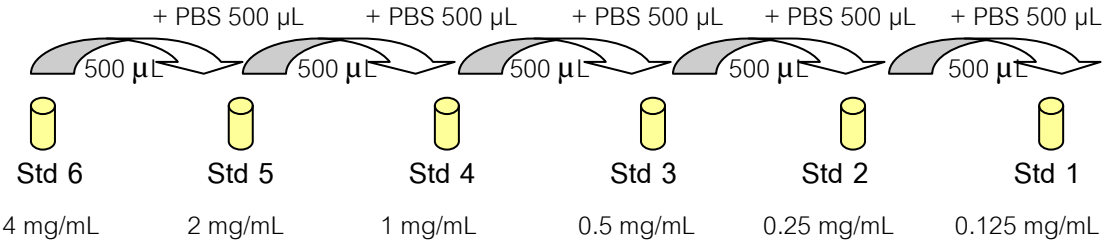
* Prepare 20 mL instead of 17 mL of working solution because it is easier for calculation and preparation.

2. Combine Reagent A with Reagent B from Pierce BCA assay kit (50:1; v/v) to prepare the working reagent

Example: total working reagent 20 mL

→ 0.4 mL of Reagent B + 19.6 mL of Reagent A

3. Prepare Bovine serum albumin (BSA) standard solution in PBS (4 mg/mL) and do the serial dilution.



BSA concentration (mg/mL)

5. Sonicate the cell suspension again (each cell pellet is suspended in 0.5 mL PBS)

6. Dilute each cell suspension with PBS (1:4; v/v)

→ 50 µL cell suspension + 150 µL PBS

7. Transfer 10 µL of each standard, blank, and unknown samples in triplicate to a 96-well microtiter plate.

8. Add 200 µL working reagent to each well

9. Mix 30 seconds and incubate on the plate shaker, 37˚C, 30 minutes

10. Read absorbance at 562 nm with a spectrophotometer (microtiter plate reader)

11. Generate a standard curve and calculate concentration of protein in samples compared with the standard. Calculated amount of protein would be corrected by multiplied by “2” for the real amount of protein in the cell pellet (dilution factor is “2” instead of “4” in this procedure because cell pellet is suspended in only 0.5 mL, but standard curve is in mg/mL. Dilution factor depends on the volume of PBS that used to suspend cell pellet, and dilution of samples before determination)

APPENDIX E: Bradford (Bio-Rad) Protein Assay

Protein contents of cell suspension were determined using Bradford Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc.) as following procedure.

1. Calculate the amount of working reagent required for the analysis in triplicate (prepare about at least 2 mL extra; use 0.2 mL of working reagent for 1 test or 1 well of 96-well microtiter plate)

Blank (n=1)

Standards (n= X; Example: X = 6 standards)

Samples (n= Y; Example: Y = 18 samples)

Total wells needed = 3(1+X+Y) = Z wells on 96-well flat bottom plate

→ 3(1+6+18) =75

Total volume of working reagent needed = (0.2 mL*Z)+2 mL

→ (0.2*75)+2 = 17 → 20 mL

* Prepare 20 mL instead of 17 mL of working solution because it is easier for calculation and preparation.

2. Dilute Dye Reagent Concentrate from Bradford Bio-Rad Protein Assay with distilled, de-ionized (DDI) water (1:5; v/v) to prepare fresh working dye reagent

Example: total working dye reagent 20 mL

→ 4 mL of Dye Reagent + 16 mL of DDI

3. Prepare six to eight concentrations of bovine serum albumin (BSA) standard solution in PBS for triplicate (0, 2.5, 5, 10, 25, 50, 75, 100 μg/mL) from a stock BSA standard (100 μg/mL), and use PBS as reagent blank

	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8
BSA concentrations (μg/mL)	0	2.5	5	10	25	50	75	100
100 μg/mL Stock BSA standard (μL)	0	5	10	20	50	100	150	200
PBS (μL)	200	195	190	180	150	100	50	0

4. Mix dialyzed LDL samples well (each sample suspended in PBS)

5. Dilute each dialyzed LDL samples with PBS (1:20; v/v),

→ 10 µL dialyzed LDL samples + 190 µL PBS

If needed, also prepare

1:50 dilution (8 µL dialyzed LDL samples + 392 µL PBS) and

1:10 dilution (20 µL diluted dialyzed LDL samples (at 1:2 dilution or very low amount of protein in samples was expected) + 180 µL PBS)

6. Transfer 50 µL of each standard, blank, and unknown samples in triplicate to a 96-well microtiter plate.

7. Add 200 µL working reagent to each well

8. Mix and incubate at room temperature for 10 minutes

9. Read absorbance at 595 nm with a spectrophotometer (microtiter plate reader)

10. Generate a standard curve (after minus blank value) and calculate concentration of protein in samples compared with the standard. Calculated amount of protein would be corrected by multiplied by dilution factor for the real amount of protein (µg/mL) in LDL samples.

APPENDIX F: Reagents for Apo B-100 Fragmentation Determination (SDS-PAGE)

1. Resolving Gel Buffer Solution, pH 8.8

Resolving gel buffer solution (1.5 M Tris-HCl, pH 8.8, 1L) can be prepared by dissolve 181.7 g of Tris-base (FW 121.1) in 750 mL of distilled, de-ionized water (DDI). Then, adjust pH of solution to be 8.8 using HCl, and add up DDI to final volume 1000 mL. Store the solution at 4°C.

2. Tris Buffer Solution, pH 6.8

Tris buffer solution (1M Tris-HCl, pH 6.8, 500 mL) can be prepared by dissolve 60.6 g of Tris-base (FW 121.1) in 350 mL of distilled, de-ionized water (DDI). Then, adjust pH of solution to be 6.8 using HCl, and add up DDI to final volume 500 mL. Store the solution at 4°C.

3. Solution for SDS-PAGE at 4% Gel Preparation

To prepare SDS-PAGE gel (4% gel), use the following formula

Chemicals needed	Volume (mL)	
	For 10 mL	For 20 mL
Distilled, de-ionized water (DDI)	6.0	11.9
30% Acrylamide/ Bis solution (Bio-Rad)	1.3	2.7
1.5 M Tris buffer, pH 8.8	2.5	5.0
10% SDS solution	0.1	0.2
10% ammonium persulfate	0.1	0.2
N,N,N',N'-tetramethyl ethylene diamine (TEMED)	0.008	0.016

Mix all chemicals together in sequent. Fill the solution into the gel casting equipment on quiet space. Be careful when apply the solution into the gel casting equipment and avoid the air bubble inside the gel. Wait until the SDS-PAGE gel set up and form well (observe from remain solution in the tube if solid gel set up). Transfer the

SDS-PAGE gel to the electrophoresis equipment. Apply the running buffer solution into the electrophoresis equipment.

4. Running Buffer Solution, pH 8.7

Buffer solution, pH 8.7 for SDS-PAGE running, 1X of this buffer contains 0.025 M Tris-base, 0.192 M Glycine, and 0.1% SDS (w/v). It can be prepared using following formula.

For 500 mL preparation of 10X running buffer solution, combine following chemical materials in 300 mL of distilled, de-ionized water (DDI)

Tris-base	0.25 M	=	15.15 g
Glycine	1.92 M	=	72.1 g
SDS	1%	=	5 g

Then, adjust pH of solution to be 8.7 using HCl if needed, and add up DDI to final volume 500 mL of 10X stock running buffer solution. Store the solution at room temperature. Dilute 1:10 with DDI before use as working running buffer solution.

5. Laemmli's SDS Denaturation Buffer Solution

For 1 mL of 2X Laemmli's SDS denaturation buffer solution for SDS-PAGE can be prepared using following formula.

1 M Tris-base solution, pH 6.8	0.125	mL
10% SDS solution	0.4	mL
Glycerol	0.2	mL
1 M DTT (1, 4-Dithiothreitol) solution	0.2	mL
Bromphenol blue dye	trace	

Mix the ingredients together and store the solution at -20˚C until used. Dilute 1 volume of this buffer with 1 volume of sample and incubate at 95ºC for 5 minutes before apply denatured samples to SDS-PAGE.

6. Coomassie Brilliant Blue G-250 Dye Reagent for Gel Staining

Dye reagent (Coomassie brilliant blue G-250) for gel staining can be prepared using following formula.

For 100 mL preparation of dye reagent, combine following chemical materials and mix well in the fume hood.

Coomassie brilliant blue G-250 dye	0.1	g
Absolute Methanol	50	mL
Distilled water (DW)	40	mL
Glacial acetic acid	10	mL

The amount of chemicals can be adjusted with the same proportion for other final volumes of solution. Keep the solution at room temperature in dark container. This dye reagent can be used for staining gel of both SDS-PAGE and agarose electrophoresis.

7. De-Staining Solution

De-staining solution for the Coomassie brilliant blue G-250 stained gel can be prepared using following formula.

For 1000 mL preparation of de-staining solution, combine following chemical materials and mix well in the fume hood.

Absolute Methanol	120	mL
Distilled water, de-ionized water (DDI)	810	mL
Glacial acetic acid	70	mL

The amount of chemicals can be adjusted with the same proportion for other final volumes of solution. Keep the solution at room temperature in dark container. This solution can be used for de-staining the Coomassie brilliant blue G-250 stained gel of both SDS-PAGE and agarose electrophoresis.

APPENDIX G: Reagents for Relative Electrophoretic Mobility - REM determination (Agarose Gel)

1. Solution for Agarose Gel Preparation (1% gel)

To prepare 150 mL of agarose gel (1% gel), use the following formula:

Agarose powder	0.75	g
1X Tris-barbital buffer solution	150	mL
(Running buffer for agarose gel electrophoresis)		

Weigh agarose powder and add it into a glass bottle. Add Tris-barbital buffer solution into the same glass bottle and warm up the glass bottle (no bottle lid) in a microwave oven at high power for 2 minutes. Let the bottle cool down for few minutes, and pour the liquid on the agarose gel casting for electrophoresis (about 1 cm thickness). Wait until the agarose gel set up and form well. Transfer the agarose gel to the electrophoresis equipment.

2. Tris-Barbital Buffer Solution, pH 8.6

Tris-barbital buffer solution, pH 8.6 for agarose gel preparation and gel running, 1X of this buffer contains 0.2 M Tris-base, 0.01 M 5,5-diethyl barbituric acid, and 0.05 M Sodium diethyl barbiturate. It can be prepared using following formula.

For 1000 mL preparation of 2X running buffer, combine following chemical materials in 700 mL of distilled, de-ionized water (DDI).

Tris-base	0.4 M	=	48.456	g
5,5-diethyl barbituric acid	0.02 M	=	3.684	g
Sodium diethyl barbiturate	0.1 M	=	20.618	g

Then, adjust pH of solution to be 8.6 using HCl if needed, and add up DDI to final volume 1000 mL of 2X stock Tris-barbital buffer solution. Store the solution at room temperature. Dilute 1:2 with DDI before use as 1X working Tris-barbital buffer solution.

APPENDIX H: Reagents for HMG-Co A Reductase Activity Experiment

1. Buffer A Solution, pH 7.2 (for Rat Liver Homogenization)

Buffer A solution, pH 7.2 for rat liver homogenization, contains 0.1 M sucrose, 0.05 M KCl, 0.04 M K_2HPO_4, and 0.03 M sodium EDTA. It can be prepared using following formula.

For 1000 mL preparation of Buffer A solution, combine following chemical materials in 700 mL of distilled, de-ionized water (DDI).

Sucrose	0.1 M	=	34.23 g
Potassium chloride (KCl)	0.05 M	=	3.73 g
Dibasic potassium phosphate (K_2HPO_4)	0.04 M	=	6.97 g
Sodium EDTA	0.03 M	=	11.17 g

Then, adjust pH of solution to be 7.2, and add up DDI to final volume 1000 mL of Buffer A solution. Store the solution at 4°C.

2. Buffer B Solution (for Re-Homogenization of Microsomes Suspension)

Buffer B is the Buffer A that contains 10 mM *dl*-dithiothreitol (DTT). For 50 mL of buffer B, add 77.1 mg of DTT into 50 mL of buffer A. Mix well and store in 4°C. Pre-heat to 37°C before use for re-homogenization of microsome suspension

3. Solution C for the Activity Assay of HMG-CoA Reductase

Solution C, pH 6.8 for the activity assay of HMG-CoA reductase, contains 0.2 M KCl, 0.16 M K_2HPO_4, 0.004 M sodium EDTA, and 0.001 M DTT. It can be prepared using following formula.

For 25 mL preparation of Solution C, combine following chemical materials in 20 mL of distilled, de-ionized water (DDI).

Potassium chloride (KCl)	0.2 M	=	372.8 mg
Dibasic potassium phosphate (K_2HPO_4)	0.16 M	=	696.7 mg
Sodium EDTA	0.004 M	=	37.2 mg
DTT	0.1 M	=	3.9 mg

Then, adjust pH of solution to be 6.8, and add up DDI to final volume 25 mL of Solution C. Store the solution at 4°C.

4. NADPH and HMG-CoA Working Solution for the Activity Assay of HMG-CoA Reductase

Prepare NADPH working solution (1 mM) by dissolving 0.005 g of NADPH in 6 mL of Solution C. Use 0.1 mL of this NADPH stock solution in 0.5 mL of the reaction mixture, then final concentration of NADPH will be 0.2 mM.

Prepare HMG-CoA stock solution (5 mM) by dissolving 5 mg of HMG-CoA in 1 mL of Solution C. Then dilute this stock solution (1:5) in Solution C to prepare HMG-CoA working solution (1 mM). Use 0.05 mL of this HMG-CoA working solution in 0.5 mL of the reaction mixture, then final concentration of HMG-CoA will be 0.1 mM

5. Reaction Mixture in Activity Assay of HMG-CoA Reductase

The activity assay of HMG-CoA reductase was performed at 37°C in a total volume of 0.5 mL. Reaction mixture began by combinding Solution C, NADPH working solution, microsome suspension (crude HMG-CoA reductase) together before adding test compounds or diluents and HMG-CoA substrate working solution (all solutions were pre-heated at 37°C). Absorbance at 340 nm was recorded before (T_0) and after mixing with HMG-CoA substrate for 5 minutes (T_5) to calculate the activity of HMG-CoA reductase. The reaction mixture which contained NADPH and other ingredients without

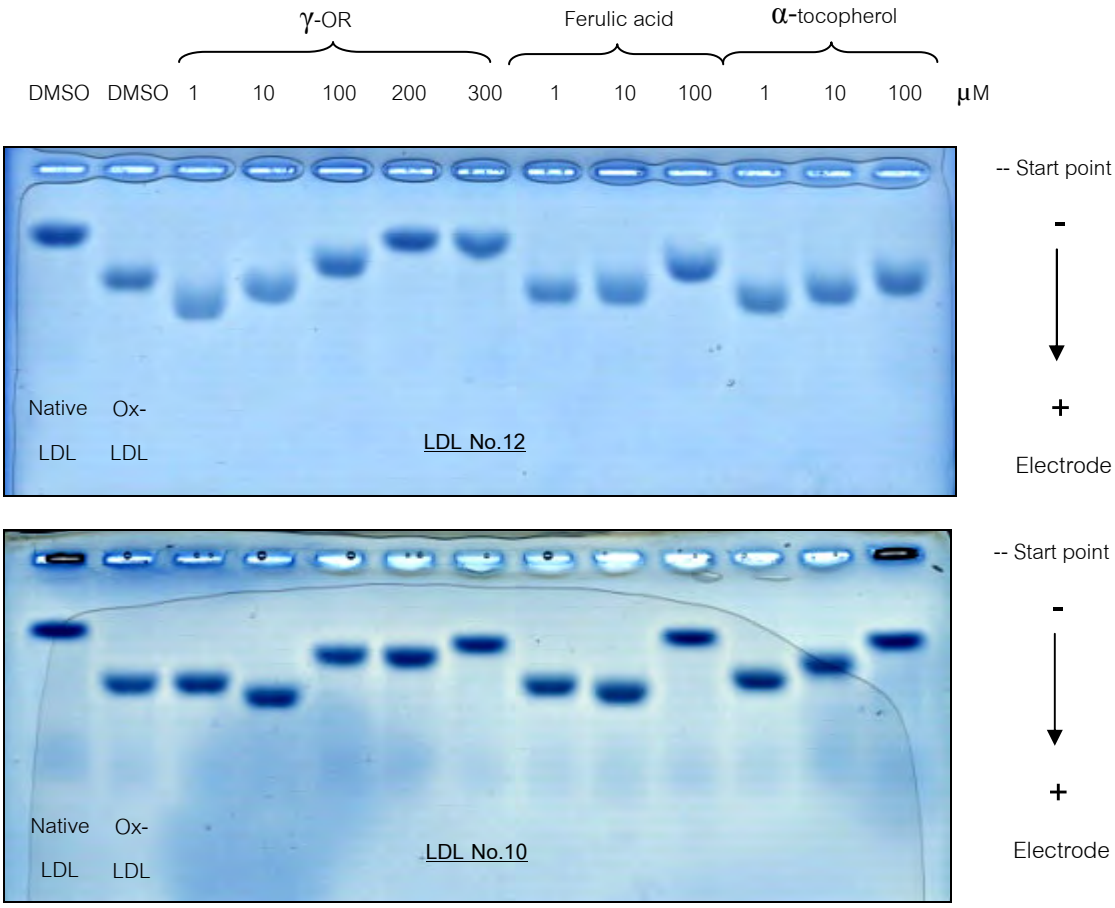
HMG-CoA substrate was used as blank. The amounts of each solution that be used in the reaction mixture in the activity of HMG-CoA reductase, are shown below.

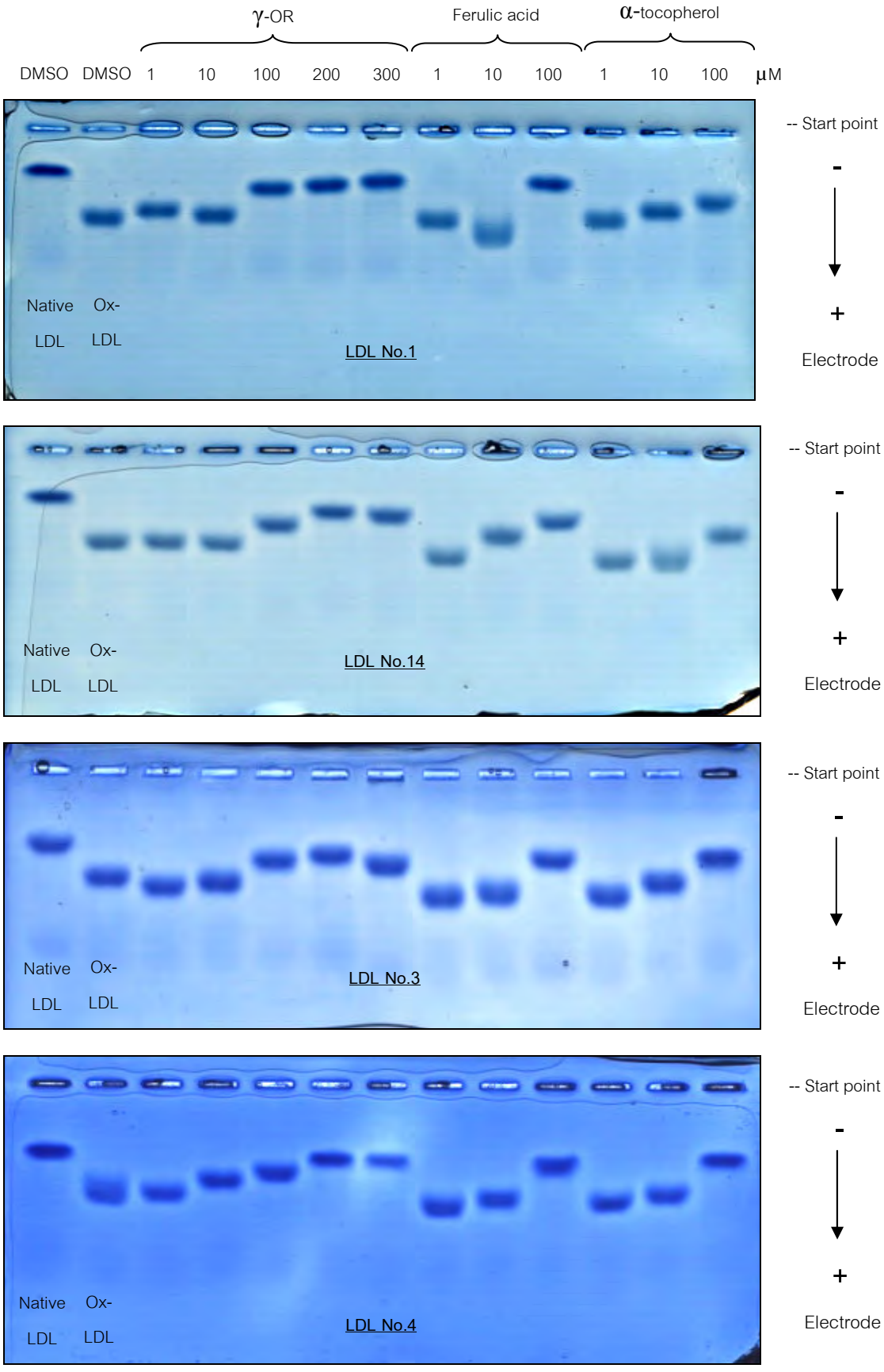
Volume (μL)	γ-OR			Ferulic acid			Simvastatin		
	blank	Control	test	blank	Control	test	blank	control	test
1) Solution C	295	245	245	295	245	245	295	245	245
2) 1 mM NADPH	100	100	100	100	100	100	100	100	100
3) Microsome suspension (crude HMG-CoA reductase)	100	100	100	100	100	100	100	100	100
4) γ-OR *	5	-	5	-	-	-	-	-	-
5) Ferulic acid *	-	-	-	5	-	5	-	-	-
6) Simvastatin *	-	-	-	-	-	-	5	-	5
7) $CHCl_3$	-	5	-	-	-	-	-	-	-
8) DMSO	-	-	-	-	5	-	-	5	-
9) 1 mM HMG-CoA substrate	-	50	50	-	50	50	-	50	50

* γ-OR (100, 200, 400 μM) = 6.03, 12.06, 24.12 mg/mL, respectively
Ferulic acid (100, 200, 400 μM) = 1.93, 3.88, 7.76 mg/mL, respectively
Simvastatin (240 μM) = 0.1 mg/mL

APPENDIX I: Agarose Gels from Relative Electrophoresis Measurement

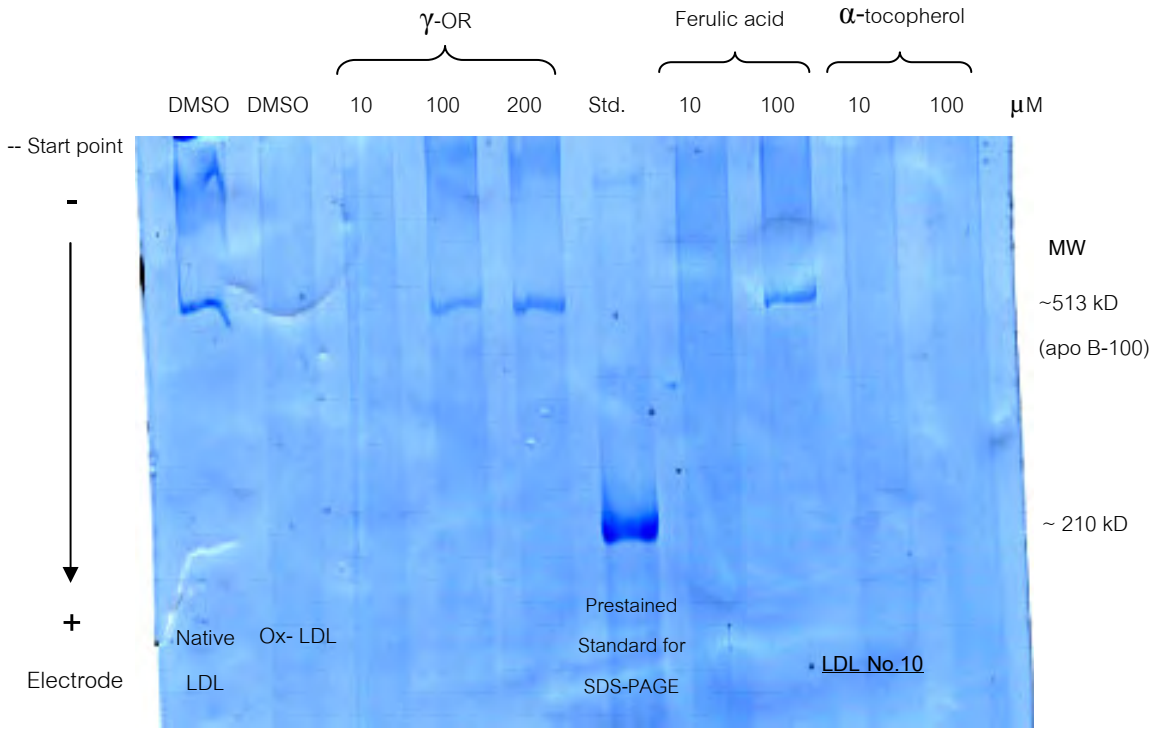
The agarose gel electrophoresis experiment in this study determined REM of native LDL and LDL (300 μg LDL protein/ mL in PBS) that be induced by 30 μM of $CuSO_4$ with various concentrations of γ-OR, ferulic acid, and α-tocopherol. Native LDL means LDL sample with the presence of DMSO, and the absence of 30 μM of $CuSO_4$ nor tested compounds. Ox-LDL means LDL sample with the presence of DMSO and 30 μM of $CuSO_4$, and the absence of tested compounds. Agarose gels from this REM experiment are shown below.

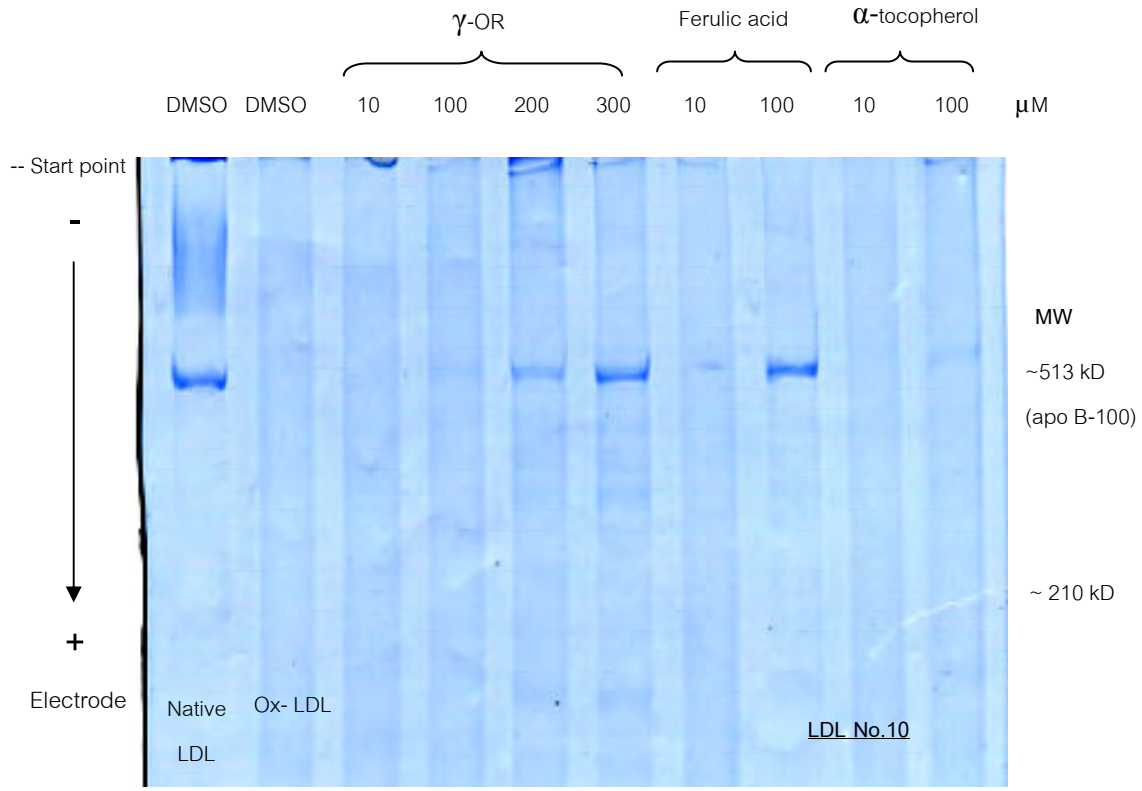


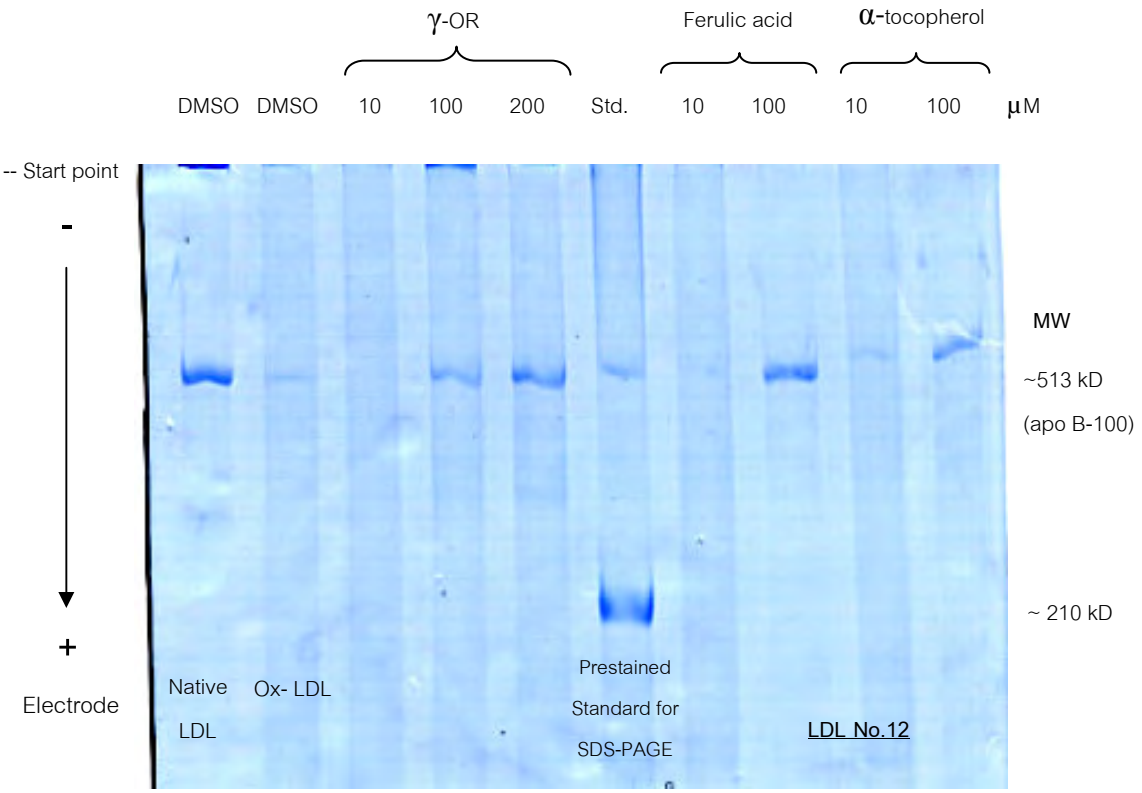

γ-OR
Ferulic acid
α-tocopherol
DMSO DMSO 1 10 100 200 300 1 10 100 1 10 100 μM
Native LDL
Ox-LDL
LDL No.1
-- Start point
-
+
Electrode
Native LDL
Ox-LDL
LDL No.14
-- Start point
-
+
Electrode
Native LDL
Ox-LDL
LDL No.3
-- Start point
-
+
Electrode
Native LDL
Ox-LDL
LDL No.4
-- Start point
-
+
Electrode

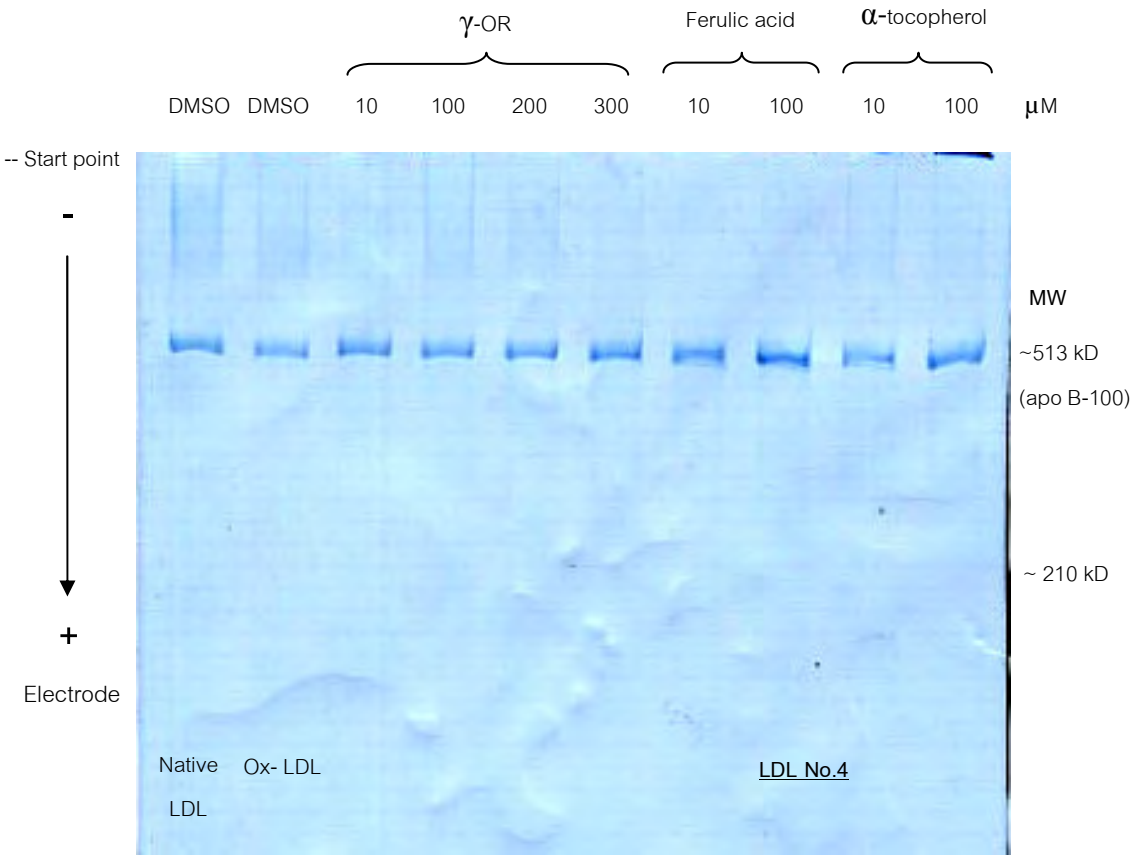
APPENDIX J: SDS-PAGE Gels from the Apo B-100 Fragmentation Experiments

The SDS-PAGE electrophoresis experiment in study determined the fragmentation of apo B-100 in native LDL and LDL (300 μg LDL protein/ mL in PBS) that be induced by 30 μM of $CuSO_4$ with various concentrations of γ-OR, ferulic acid, and α-tocopherol. Native LDL means LDL sample with the presence of DMSO, and the absence of 30 μM of $CuSO_4$ nor tested compounds. Ox-LDL means LDL sample with the presence of DMSO and 30 μM of $CuSO_4$, and the absence of tested compounds. SDS-PAGE gels from the experiment are shown below.




γ-OR
Ferulic acid
α-tocopherol
DMSO DMSO 10 100 200 300 10 100 10 100 μM
-- Start point
-
+
Electrode
Native LDL
Ox- LDL
LDL No.10
MW
~513 kD
(apo B-100)
~ 210 kD


γ-OR
Ferulic acid
α-tocopherol
DMSO DMSO 10 100 200 Std. 10 100 10 100 μM
-- Start point
-
+
Electrode
Native LDL
Ox- LDL
Prestained Standard for SDS-PAGE
LDL No.12
MW
~513 kD
(apo B-100)
~ 210 kD


γ-OR
Ferulic acid
α-tocopherol
DMSO DMSO 10 100 200 300 10 100 10 100 μM
-- Start point
-
+
Electrode
MW
~513 kD
(apo B-100)
~ 210 kD
Native LDL
Ox- LDL
LDL No.4

BIOGRAPHY

Mrs. Kittana Mäkynen, previously Miss Kittana Chanda, was born on September 17, 1979 in Ratchaburi, Thailand. She graduated with a Bachelor degree of Science in Medical Technology from the Faculty of Medical Technology, Mahidol University, Thailand in February 17, 2000. In June 2000, she continued her higher education immediately in the Master of Science Program in Nutrition at the Faculty of Medicine, Ramathibodi Hospital, Mahidol University. During her study in the Master of Science Program, she received financial support from the scholarship under The Commission on Higher Education Staff Development Project, Ministry of Education, Thailand, from June 2001 to May 2003. She graduated with a Master degree of Science in Nutrition from Mahidol University in May 6, 2004.

During the period from June 2004 to May 2007, she received a scholarship under The Commission on Higher Education Staff Development Project scheme for her study in Ph.D. Program in Biomedical Sciences at Chulalongkorn University, Thailand. The scholarship enabled her to spend one year for her research as a visiting scholar at Department of Human Nutrition, The Ohio State University, Columbus, OH, USA.

On September 9, 2009 she married Mr. Sami Olavi Mäkynen in Kemi, Finland.

After her Doctoral degree graduation, she will work as a lecturer in the Nutrition and Dietetics Program at the Faculty of Allied Health Sciences, Chulalongkorn University according to the scholarship contract with The Commission on Higher Education, Ministry of Education, Thailand.