อิทธิพลของภาวะไตรเอซิลกลีเซอรอลสูงในเลือดหลังมื้ออาหารต่อการเปลี่ยนแปลงของอะโปโปรตีนบนไลโปโปรตีนชนิดที่มีไตรเอซิลกลีเซอรอลเป็นองค์ประกอบหลักในผู้ป่วยเบาหวานชนิดที่ 2 ที่มีภาวะดื้อต่ออินซูลินต่างกันโดยแมสสเปกโทรเมทรี

นางสาวศิริพร แสงสุธรรม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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THE INFLUENCE OF POSTPRANDIAL HYPERTRIACYLGLYCEROLEMIC STATE OF TYPE 2 DIABETES WITH VARIED INSULIN RESISTANCE ON ALTERATIONS OF APOPROTEINS IN TRIACYLGLYCEROL-RICH LIPOPROTEINS STUDY USING MASS SPECTROMETRY

Miss Siriporn Sangsuthum

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Thesis Title	THE INFLUENCE OF POSTPRANDIAL HYPERTRIACYLGLYCEROLEMIC STATE OF TYPE 2 DIABETES WITH VARIED INSULIN RESISTANCE ON ALTERATIONS OF APOPROTEINS IN TRIACYLGLYCEROL-RICH LIPOPROTEINS STUDY USING MASS SPECTROMETRY
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Field of Study	Biomedical Sciences
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ศิริพร แสงสุธรรม : อิทธิพลของภาวะไตรเอซิลกลีเซอรอลสูงในเลือดหลังมื้ออาหารต่อการเปลี่ยนแปลงของอะโปโปรตีนบนไลโปโปรตีนชนิดที่มีไตรเอซิลกลีเซอรอลเป็นองค์ประกอบหลักในผู้ป่วยเบาหวานชนิดที่ 2 ที่มีภาวะดื้อต่ออินซูลินต่างกันโดยแมสสเปกโทรเมทรี. (THE INFLUENCE OF POSTPRANDIAL HYPERTRIACYLGLYCEROLEMIC STATE OF TYPE 2 DIABETES WITH VARIED INSULIN RESISTANCE ON ALTERATIONS OF APOPROTEINS IN TRIACYLGLYCEROL-RICH LIPOPROTEINS STUDY USING MASS SPECTROMETRY) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร.วินัย ดะห์ลัน, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ.ดร.พลกฤษณ์ แสงวณิช, อ.ดร.ระวีนันท์ มิ่งภัคนีย์, 149 หน้า.

เป็นที่ทราบกันดีว่าภาวะดื้ออินซูลินเป็นตัวการสำคัญในการทำให้เกิดภาวะไขมันผิดปกติ โดยเฉพาะอย่างยิ่งภาวะไตรเอซิลกลีเซอรอลสูงในผู้ป่วยเบาหวานอย่างไรก็ตามในผู้ป่วยเบาหวานชนิดที่ 2 ที่มีการควบคุมระดับน้ำตาลเป็นอย่างดี และมีระดับไตรเอซิลกลีเซอรอลอยู่ในเกณฑ์ปกติ ภาวะไตรเอซิลกลีเซอรอลในเลือดสูงจะพบได้ในภาวะหลังมื้ออาหารเท่านั้น ซึ่งในการวิจัยครั้งนี้ ต้องการศึกษาอิทธิพลของภาวะดื้ออินซูลินต่อไลโปโปรตีนโปรไฟล์และอะโปโปรตีนในไลโปโปรตีนชนิดความหนาแน่นต่ำ (VLDL) ในผู้ป่วยเบาหวานชนิดที่ 2 เทียบกับกลุ่มควบคุม 10 คน แบ่งผู้ป่วยเบาหวานชนิดที่ 2 ที่มีค่า HbA$_1$C น้อยกว่า 8% ออกเป็น 2 กลุ่มตามค่า HOMA-IR จะได้กลุ่ม LIR ที่มีค่า HOMA-IR น้อยกว่า 2.5 จำนวน 5 คน, กลุ่ม HIR ที่มีค่า HOMA-IR ระหว่าง 2.5 และ 5.0 จำนวน 6 คน ผู้เข้าร่วมโครงการวิจัยทุกคนจะต้องอดอาหารมาอย่างน้อย 12 ชั่วโมงก่อนที่จะรับประทานอาหารที่มีไขมันสูงเป็นองค์ประกอบ ทำการเจาะเลือดทั้งก่อนกินอาหารและหลังอาหาร 4 ชั่วโมง เพื่อนำไปวิเคราะห์หาค่ากลูโคสอินซูลินไขมันในเลือด และศึกษาวิเคราะห์ไลโปโปรตีนโปรไฟล์ในการทดลองที่ 1 พบว่าระดับของไตรเอซิลกลีเซอรอลในภาวะหลังอาหารจะเพิ่มขึ้นมากที่สุดในกลุ่ม HIR โดยเพิ่มในส่วนของไคโลไมครอน, VLDL และHDL สำหรับการทดลองที่ 2 เตรียม VLDL จากพลาสมารวมของแต่ละกลุ่มตัวอย่างทั้งก่อนและหลังมื้ออาหาร และนำไปวิเคราะห์ต่อด้วย 2D-PAGE และแมสสเปกโทรเมทรี ผลการทดลองที่ได้คือสามารถวิเคราะห์บ่งชนิดของโปรตีนได้ถึง 14 ชนิด รวมถึงไอโซฟอร์มของโปรตีนใน VLDL นอกจากนี้ยังตรวจพบโปรตีน transthyretin (TTR) ซึ่งไม่เคยตรวจพบใน VLDL มาก่อน และจากการเปรียบเทียบการเปลี่ยนแปลงของอะโปโปรตีนบน VLDL ระหว่างก่อนและหลังมื้ออาหารในกลุ่มตัวอย่างเดียวกัน พบการเปลี่ยนแปลงอย่างมีนัยสำคัญของ apoC-II, apoC-III, apoE, apoA-I ในทุกกลุ่มตัวอย่าง และมีเพียงกลุ่ม HIR เท่านั้นที่พบการเปลี่ยนแปลงของ TTR เมื่อทำการเปรียบเทียบการเปลี่ยนแปลงของอะโปโปรตีนบน VLDLระหว่าง LIR, HIR กับกลุ่มควบคุมในภาวะก่อนอาหาร พบการเปลี่ยนแปลงอย่างมีนัยสำคัญ ของ apoA-I, apoA-IV, apoC-II ในทั้ง LIR และ HIR ในขณะที่พบการเปลี่ยนแปลงของ apoC-III, apoE, TTR เฉพาะกลุ่ม HIR เท่านั้น และเมื่อทำการเปรียบเทียบระหว่างกลุ่มในภาวะหลังมื้ออาหารพบการเปลี่ยนแปลงอย่างมีนัยสำคัญของapoA-I, apoC-II, apoC-III, apoE, TTR ในทั้ง LIR และ HIR เทียบกับกลุ่มควบคุม สรุปได้ว่าภาวะดื้อต่ออินซูลินทำให้เกิดการเปลี่ยนแปลงของ VLDL ทั้งส่วนลิปิดและโปรตีน การเปลี่ยนแปลงดังกล่าวอาจก่อความผิดปกติแก่เมตาบอลิสมของไลโปโปรตีนในผู้ป่วยเบาหวาน

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ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม ระวีนันท์ มิ่งภัคนีย์

4689717820 : MAJOR BIOMEDICAL SCIENCES
KEYWORDS : apolipoprotein / proteomics / type2 diabetes / mass spectrometry / VLDL

SIRIPORN SANGSUTHUM: THE INFLUENCE OF POSTPRANDIAL HYPERTRIACYLGLYCEROLEMIC STATE OF TYPE 2 DIABETES WITH VARIED INSULIN RESISTANCE ON ALTERATIONS OF APOPROTEINS IN TRIACYLGLYCEROL-RICH LIPOPROTEINS STUDY USING MASS SPECTROMETRY. THESIS ADVISOR: ASSOCIATE PROFESSOR WINAI DAHLAN, Ph.D., THESIS CO-ADVISOR: ASSOCIATE PROFESSOR POLKIT SANGVANICH, Ph.D., RAVEENAN MINGPAKANEE, Ph.D., 149 pp.

It is known that insulin resistance plays role in diabetic dyslipidemia especially hypertriacylglycerolemia (HTAG). However, in good metabolic controlled type2 diabetes mellitus who have normal fasting triacylglycerol level, the HTAG can observed only in postprandial state. In this research, the influence of insulin resistance on lipoprotein profiling and apoprotein of triacylglycerol-rich lipoprotein especially very low-density liprotein (VLDL) were studied. Eleven type2 diabetic subjects with moderated metabolic control (HbA1c <8%), were classified as lower insulin resistance, LIR (HOMA-IR<2.5), n=5 and higher insulin resistance, HIR (HOMA-IR 2.5-5.0), n=6 comparable to 10 nondiabetic controls. After at least 12 hours of fasting, subjects consumed a fat-rich meal. Before and the 4 hours after the meal, blood samples were taken for determination of glucose, insulin, lipids and lipoproteins in Experiment I. The increasing of postprandial triacylglycerol was found in chylomicron VLDL and HDL fractions especially in HIR group. For Experiment II, VLDL was isolated using sequential ultracentrifugation, delipidated and separated with 2D-PAGE and identified using ESI-IT, LC-MS/MS and MALDI-TOF MS. In total 26 protein spots representing 14 proteins including novel protein, transthyretin (TTR) with their isoforms were identified in VLDL. The comparisons of 14 proteins were analyzed in 6 pooled VLDL between fasting state and postprandial state within the same group as well as among different groups. The significant alterations compared between fasting and postprandial state of VLDL proteins (p<0.05) in all groups were observed among apoC-II, apoC-III, apoE, apoA-I, except for TTR which was found only in HIR one. In fasting state, the significant changes of apoA-I, apoA-IV, and apoC-II were observed in LIR and HIR, whereas of apoC-III, apoE and TTR were observed in HIR in comparison to controls. In addition, significant modifications were found in apoA-I, apoC-II, apoC-III, apoE, and TTR of postprandial state compared to controls. These findings illustrate that insulin resistance is not only associated with increased VLDL triacylglycerol levels but also with alterations in VLDL protein composition which possibly affect the lipoprotein metabolism in diabetic patients.

Field of Study : Biomedical Sciences Student's Signature Siriporn Sangsuthum

Academic Year : 2009 Advisor's Signature

Co-Advisor's Signature

Co-Advisor's Signature Raveenan Mingpakanee

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Abs	absorbance
ACN	acetronitrile
ApoA-I	apoproteinA-I
ApoA-II	apoproteinA-II
ApoA-IV	apoproteinA-IV
ApoB-48	apoproteinB-48
ApoB-100	apoproteinB-100
ApoC-I	apoproteinC-I
ApoC-II	apoproteinC-II
ApoC-III	apoproteinC-III
ApoC-IV	apoproteinC-IV
ApoE	apoproteinE
BSA	bovine serum albumin
C-terminus	COOH terminus of peptide
CAD	coronary artery disease
CD	circular dichroism
CETP	cholesteryl ester transfer protein
CHCA	α−cyano-4-hydroxy-cinnamic acid
$CHCl_3$	chloroform
DM2	type2 diabetes
DTT	dithiothreitol
2-DE	two-dimensional electrophoresis
EDTA	ethylenediamine tetraacetic acid
ESI	electrospray ionization
FA	formic acid
HDL	high-density lipoprotein
HIR	higher insulin resistance subjects
HL	hepatic lipase
HOMA-IR	homeostatic model assessment-insulin resistance
HPLC	high performance liquid chromatography

ICAM-1	intercellular adhesion molecule-1
IDL	intermediate-density lipoprotein
IEF	isoelectric focusing
IT	ion trap mass analyzer
KBr	potassium bromide
kV	kilovolts
LCAT	lecitin-cholesterol acyltransferase
LDL	low-density lipoprotein
LIR	lower insulin resistance subjects
LPL	lipoprotein lipase
MALDI	matrix-assisted laser/desorption ionization
MCP-1	monocyte chemotactic protein-1
MeOH	methanol, methyl alcohol
m/z	mass-to-charge ratio
min	minute
ml	millilitre
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MW	molecular weight
NaCl	sodium chloride
N-terminus	NH_3 terminus of peptide
pI	isoelectric point
PMF	peptide mass fingerprint
PON1	paraoxonase I
PTM	post-translational modification
PUFA	polyunsaturated fatty acid
RF	radio frequency
Rf	migration distances
rpm	round per minute
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

TAG	triacylglycerol
TC	total cholesterol
TEM	transmission electron microscope
TNF	tumor necrosis factor
ToF	time-of-flight mass analyzer
TRLs	triacylglycerols-rich lipoproteins
UV	ultraviolet
VCAM-1	vascular cell adhesion molecule-1
VLDL	very low–density lipoprotein
w/v	weight by volume
WHO	world health organization
μg	microgram
μl	microlitre
μM	micromolar

CHAPTER I

INTRODUCTION

World Health Organization (WHO) report in November 2009 showed more than 220 million people worldwide have diabetes, which increase from 171 million in year 2000 and estimate to double by the year 2030 (Wild *et al.*, 2004). Ninety percents of people with diabetes around the world is type2 diabetes. The common effect of uncontrolled diabetes and over time leads to serious damage to many of the body's systems, especially the nerves and blood vessels. Diabetes has a three to fourfold increase in risk of heart disease and stroke compare to non-diabetic people. Fifty percent of people with diabetes die of cardiovascular disease, primarily heart disease and stroke (World Health Organization, 2009: online).

Diabetes has been considered to have the high risk of developing coronary heart disease (CHD) which directly relate to the combination of dyslipidemia characterized by high level of fasting plasma triacylglycerol (TAG), and lead to low levels of high-density lipoproteins (HDL) and predominance of small dense low-density lipoproteins (LDL) cholesterol (Ginsberg *et al.*, 2005; Kreisberg, 1998). As known, HDL functions as reverse cholesterol transport and also have protective property in atherosclerosis such as anti-oxidative, anti-inflammatory and anti-thrombotic properties (Assmann *et al.*, 2004; Movva and Rader, 2008), and LDL are related directly to the development of atherosclerosis especially small dense LDL have more atherogenic potential, readily oxidized than the larger and less dense LDL (de Graaf *et al.*, 1991). The development of the above dyslipidemia in diabetes is considered as a result of insulin resistance especially at the beginning of the abnormality chain, overproduction of VLDL and hypertriacylglycerolemia (Ginsberg, 2000)

Besides the high level fasting triacylglycerol, postprandial hypertriacylglycerolemia also is a high risk conditions for cardiovascular disease and become a stronger relationship with cardiovascular disease than fasting triacylglycerol

level (Bansal *et al.*, 2007). In addition, the postprandial hypertriacylglycerolemia also found in myocardial infarction patients who have normal fasting TAG level (Tsunoda *et al.*, 2004). The correlation between postprandial hypertriacylglycerolemia and cardiovascular risk due to excessive postprandial triacylglycerolemia modifies properties of both LDL and HDL towards predominance of more atherogenic subclass profiles (Taskinen, 2005), including the accumulation of atherogenic VLDL, chylomicrons and their remnants commonly termed “triacylglycerol-rich lipoproteins (TRLs)”. TRLs remnants can penetrate the endothelium, promote endothelial dysfunction and cause foam cell formation (Mamo *et al*., 1998; Ceriello *et al.*, 2006).

The dyslipidemia in type2 diabetes as mention above not only occurs from plasma lipoproteins level, it occurs in apolipoproteins as well. Because apolipoproteins or “apo” in brief are the composition of lipoproteins and play critical roles in the lipoprotein metabolism, both regulation of metabolism and acting as ligands for lipoprotein receptors. So the alterations of apolipoproteins lead to the defective of lipoprotein metabolism.

To study the apolipoprotein, normally immunoaffinity method is used because it is a specific and high sensitivity method however this method can not detect the modification of protein, unlike mass spectrometry. Nowadays, mass spectrometry (MS) becomes a modern technique used in proteins research, especially for proteins identification, modification including proteins polymorphism (Ito *et al.*, 1989; Bondarenko *et al*., 1999). Moreover, the combination of MS with two-dimensional gel electrophoresis (2-DE), proteomics tool, has shown as the powerful tool to study of quantitative changes of expression levels includes modification in protein.

Recently, proteomics tool was used to study whole proteins in lipoprotein such as very low-density lipoprotein (VLDL) (Mancone *et al.*, 2007), low-density lipoprotein (LDL) (Karlsson *et al*., 2005), high-density lipoprotein (HDL) (Karlsson *et al.*, 2005; Rezaee *et al*., 2006). By this technique, the novel protein in addition to apolipoproteins could be found and open the new frame of lipoprotein research. Recently, proteomics tool was applied in clinical research for study of LDL protein profiling from obese subjects (Karlsson *et al*., 2009).

Our study is divided into two experiments. First, study on the influence of insulin resistance on the lipoprotein profiling including LDL particles size of type2 diabetes compare to non-diabetic subjects. The second is a study of the insulin resistant influence on the alteration of apoproteins of VLDL between type2 diabetes and non-diabetic subjects in postprandial state.

Experiment I: Study on the influence of insulin resistance on diabetic lipoprotein profiling including small dense LDL particles size.

Purpose: To study the effects of insulin resistance on lipoprotein profiling both fasting and postprandial states include the small dense LDL particles size. The results were compared between type2 diabetic subjects with varied state of insulin resistance and non-diabetic subjects for better understanding the influence of insulin resistance on lipoprotein metabolism in type2 diabetic subjects.

Experiment II: Study on the influence of insulin resistance on the alteration of apolipoproteins of very low density lipoprotein (VLDL) between type2 diabetes and non-diabetic subjects in postprandial state.

Purpose: To study the effects of insulin resistance on apolipoprotein profiling on VLDL both fasting and postprandial states. The proteomic tools were used to investigate the role of insulin resistance on protein profiling of VLDL between type2 diabetic subjects with varied state of insulin resistance and non-diabetic subjects in postprandial state that has not been elucidated for better understanding of lipoprotein metabolism in type2 diabetic subjects.

The study of Experiment I begins with determination of clinical blood chemistry such as blood sugar, total cholesterol, HDL cholesterol, plasma triacylglycerol, plasma insulin level, and HbA1C from 12 hours fasting and 4 hours postprandial EDTA plasma of non-diabetic subjects as a control group and type2 diabetes who has different insulin resistance characterized from homeostasis model assessment insulin resistance, HOMA-IR (Matthews *et al.*, 1985) as a higher insulin resistance group, HOMA-IR between 2.5 to 5.0 and a lower insulin resistance group,

HOMA-IR less than 2.5. Lipoproteins fractions were prepared using sequential ultracentrifugation and further analyzed for cholesterol and triacylglycerol. The particles size of small dense LDL from fasting and postprandial were determined using gradient non-denaturing polyacrylamide electrophoresis.

The different results from baseline and postprandial were analyzed with paired-samples *t*-test. Differences among 3 groups were performed by analysis of variance (ANOVA) with Duncan's New Multiple Range Test. $P < 0.05$ was considered to be statistically significant.

Experiment II, proteomics tool base on two-dimensional electrophoresis (2-DE) and mass spectrometry both MALDI-ToF and LC-MS/MS (ESI-IT) was optimized to analyzed VLDL associated proteins and further analyzed for apolipoproteins profile of VLDL from fasting and 4 hr postprandial plasma of diabetic and non-diabetic subjects.

The different amount of VLDL proteins calculated from the mean relative amount (%) of each protein between non diabetes and both of diabetic groups from fasting and postprandial were analyzed with *t*-test. $P < 0.05$ was considered to be statistically significant.

CHAPTER II

LITERATURE REVIEW

2.1 Lipoproteins

Lipoproteins are soluble complexes particles that transport lipids in the circulation. Lipoproteins are spherical shape and vary in diameter, depending on the particular proteins and lipids. The structures of the various lipoproteins appear to be similar. Each of the lipoprotein classes compose of hydrophobic lipid core which contains primarily triacylglycerol and cholesterol esters, surrounded by hydrophilic layer of phospholipids, cholesterol, and specialized protein, called apolipoproteins or apoproteins and apo in brief (Figure 2.1). These proteins facilitate lipid solubilization and help to maintain the structural integrity of lipoproteins. They also serve as ligands for lipoprotein receptors and regulate the activity of lipoprotein metabolic enzymes.

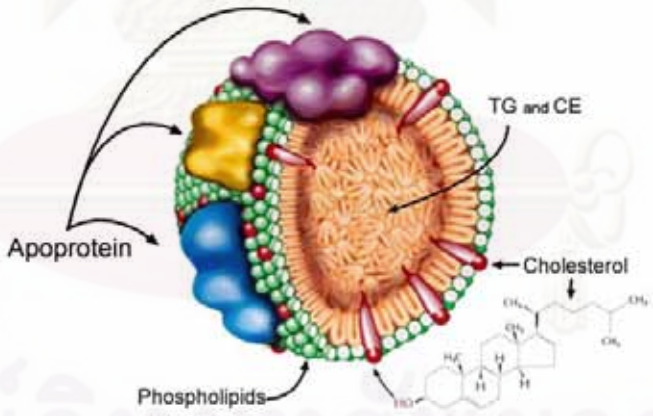


Figure 2.1 General structure of a lipoprotein particle. The spherical particle consists of a neutral lipids core surrounded by a surface of phospholipids, cholesterol and apolipoproteins (http://www.peprotech.co.kr)

2.1.1 Classification and Functions of Lipoproteins

Lipoproteins are classified into five major types on the basis of their density, as determined by centrifugation, and physical properties (Table 1).

Table 2.1 Composition and density of human lipoproteins (Voet and Voet, 1995)

	Chylomicron	**VLDL**	**IDL**	**LDL**	**HDL**
Density (g/ml)	< 0.95	0.95-1.006	1.006-1.019	1.019-1.063	1.063-1.210
Diameter (nm)	100-1,000	35-50	28-35	20-26	7-12
Component (% dry weight)					
Protein[a]	2	8	15	22	40-55
Triacylglycerol[b]	86	55	31	6	4
Free cholesterol[a]	2	7	7	8	4
Cholesterol esters[b]	3	12	23	42	12-20
Phospholipids[a]	7	18	22	22	25-30
Main apoproteins	apoB-48, C-II, A-I	apoB-100, C-II, E	apoB-100, C-II, E	apoB-100	apoA-I, A-II

[a] Surface components

[b] Core lipids

Chylomicrons are synthesized in the intestine. They are the largest and least dense of the plasma lipoprotein classes. Their main function is to transport dietary triacylglycerol from the intestine to tissues throughout the body. As such, their predominant core lipid is triacylglycerol. Chylomicrons also provide a vehicle for the delivery of dietary cholesterol from the intestine to the liver. The main apoprotein in chylomicrons is apoB-48 (Kane, 1983). They also contain substantial amounts of the apoproteinC. Chylomicrons are catabolised to chylomicron remnants, which also contain apoE. The apoproteinE in chylomicron remnants is transferred from HDL during chylomicron catabolism (Havel *et al.*, 1973).

Very Low Density Lipoproteins (VLDL) is synthesized in the liver. Their main function is to transport triacylglycerol to tissues throughout the body. Thus, like chylomicrons, they have a core which is rich in triacylglycerol. They also have an additional function as transport vehicles for cholesterol in a process which delivers hepatic cholesterol into the plasma. However, VLDL is smaller and denser than chylomicrons. They also contain apoB-100, rather than apoB-48, as their main apoprotein (Kane, 1983). Most VLDL contains apoE and apoC (McConathy and Alaupovic, 1986).

Intermediate Density Lipoproteins (IDL) is catabolic products of VLDL and intermediates in the pathway in which VLDL are converted to LDL. IDL are smaller and denser than VLDL and their core contains approximately equal amounts of triacylglycerol and cholesteryl esters. The main apoproteins in IDL are apoB-100 and apoE (Kane, 1983; McConathy and Alaupovic, 1986). IDL are normally present in plasma at low concentration and are of uncertain function.

Low Density Lipoproteins (LDL) is catabolic products of IDL. They are what remain after IDL have lost most of their triacylglycerol and all of their apoproteins, apoC and apoE. LDL is smaller and denser than IDL and their core contains predominantly cholesteryl esters. Each LDL particle contains a single molecule of apoB-100 as its sole apolipoprotein constituent (Kane, 1983). The LDL fraction comprises a number of particles of varying size and density (Krauss and Burke, 1982). One of the major functions of LDL is the delivery of cholesterol to cells.

High Density Lipoproteins (HDL) is the smallest, most dense plasma lipoproteins. They originate in the liver and intestine as lipid-poor particles which rapidly become cholesterol-enriched as they acquire cholesterol from tissues throughout the body. The predominant HDL core lipid is cholesteryl ester. The main HDL apoproteins are apoA-I and apoA-II which account for more than 90% of the apoproteins in HDL. HDL also contains smaller amounts of apoA-IV, apoC and apoE. HDL may be subdivided on the basis of size and density into smaller and denser HDL3 and larger and less dense HDL2 (Blanche *et al.*, 1981). They may be separated

on the basis of apoprotein composition into two main subpopulations: one containing apoA-I without apoA-II (A-I HDL) and the other containing both apoA-I and apoA-II (A-I/A-II HDL) (Cheung and Albers, 1984). A major function of HDL is to act as a recipient of the cholesterol which is transferred from extrahepatic tissues into the plasma via the pathway termed reverse cholesterol transport. HDL also has protective property in atherosclerosis such as anti-oxidative mechanism (Assmann *et al.*, 2004).

2.1.2 Apolipoproteins

"Apolipoproteins" or **"apoproteins"** are the protein components of lipoproteins. The major apoproteins found in human lipoproteins including their properties are summarized in Table 2.

The structure and function of these apoproteins has been intensely studied in the past decade, and most of the apoproteins have been sequenced. The synthesis of the apoproteins takes place on ribosomes that are bound to endoplasmic reticulum of the liver or intestinal cells. The apoproteins may be classified into two types: the non-exchangeable and the exchangeable apoproteins. ApoB-100 and apoB-48 are non-exchangeable apoproteins. They are very large and water-insoluble proteins that are assembled with lipids on lipoprotein particles. These non-exchangeable apoproteins circulate bound to the same lipoprotein particle through various metabolic transformations in plasma, until they are cleared, as lipoproteins, via specific receptors. In contrast, the exchangeable apoproteins - apoA-I, apoA-II, apoC, apoE- have much smaller molecular masses than apoB-100 or apoB-48. They are water-soluble and associate rather weakly with lipoproteins. Hence, they readily transfer between lipoprotein particles via the aqueous phase.

Table 2.2 Apoproteins of the human plasma lipoproteins (Voet and Voet, 1995)

Apoproteins	***Molecular weight***	***Characteristics***
A-I	28,300	Major protein in HDL; Activates LCAT[a]
A-II	17,400	Major protein in HDL; Activates hepatic lipase
B-48	241,000	Found exclusively in chylomicrons; Cholesterol clearance
B-100	513,000	Major protein in LDL; Cholesterol clearance
C-I	7,000	Found in chylomicrons; Inhibits LPL[b]
C-II	10,000	Found primarily in VLDL; Activates LPL
C-III	9,300	Found primarily in chylomicrons, VLDL and HDL; Inhibits LPL
E	33,000	Found in VLDL, LDL, and HDL; Cholesterol clearance

[a] LCAT; Lecithin-cholesterol acyl transferase

[b] LPL; Lipoprotein lipase

All apoproteins are monomers except apoA-II, which is a disulfide-linked dimer. Circular dichroism (CD) measurements indicated that apoproteins have a high helix content, which increased when they are incorporated in lipoproteins. Apoproteins alpha helices float on phospholipid surfaces of lipoproteins, much like logs on water. The phospholipids are arrayed with their charged groups bound to oppositely charged residues on the polar face of the helix and with the first few methylene groups of their fatty acid residues in hydrophobic association with the nonpolar face of the helix (Orten *et al.*, 1982; Voet and Voet, 1995).

ApoproteinA-I is the major apoprotein of HDL. The main function is promoting the efflux of cholesterol from cells, and also activates LCAT. ApoA-I also has anti-oxidant, anti-inflammatory properties and inhibits atherosclerotic lesion formation at the early foam cell stage in mice (Dansky *et al.*, 1999). Decreased concentrations of apoA-I in plasma correlate to increase risk of premature coronary

artery disease and apoA-I concentrations also decrease in CAD patients (Campos *et al.*, 1992).

ApoproteinA-II is the second major apoprotein of HDL. ApoproteinA-II activates hepatic lipase (HL) and seems to function to regulate the size of the HDL particles, but the main function has still not clear. ApoproteinA-II concentrations in plasma are inversely associated with the development of premature cardiovascular disease. Transgenic mice over expressing human apoA-II display high VLDL and low HDL levels (Boisfer *et al.*, 2002). Humans and mice lacking apoA-II have reduced levels of plasma cholesterol (Weng and Breslow, 1996).

Apoprotein A-IV may have a role in chylomicrons and VLDL secretion and catabolism. Required for efficient activation of lipoprotein lipase by ApoC-II; potent activator of LCAT. ApoA-IV is a major component of HDL and chylomicrons and has positive regulation effect on lipoprotein lipase activity and facilitate of cholesterolyl ester transfer protein (CETP) activity. (Verges, 1995 and ExPASy Proteomics Server : online).

Apoprotein A-V is induced in early phase of liver regeneration and a minor apoprotein mainly associated with HDL and to a lesser extent with VLDL, may also be associated with chylomicrons. Important determinant of plasma triglyceride (TG) levels by both being a potent stimulator of apo-CII lipoprotein lipase (LPL) TG hydrolysis and an inhibitor of the hepatic VLDL-TG production rate (without affecting the VLDL-apoB production rate). Activates poorly lecithin:cholesterol acyltransferase (LCAT) and does not enhance efflux of cholesterol from macrophages. Defects in apoA-V are a cause of susceptibility to familial hypertriglyceridemia (ExPASy Proteomics Server : online).

ApoproteinB in human can be separated into two forms, apoB-48 and apoB-100 which are synthesized from different source. ApoB-48 is synthesized by intestine, while apoB-100 is synthesized by liver. ApoB-48 contains 48% of the N-terminal sequence of apoB-100. While, apoB-48 is a major structure protein in chylomicrons, apoB-100 is a major structure protein in LDL and also found in VLDL.

ApoproteinB-100 interacts with B/E receptor and causes the LDL particle to be internalized and degraded, thus allowing the LDL to deliver its cholesterol to the cell or cholesterol clearance.

ApoproteinC-I inhibits lipoprotein lipase and the concentration of plasma apoC-I is a predictive of the plasma triacylglycerol level and also of early atherosclerosis. Transgenic mice over expressing human apoC-I increased VLDL particle production and LPL inhibition (Westerterp *et al.*, 2006). The apoproteinC-I content of postprandial TRLs is an independent risk factor for early atherosclerosis in human (Björkegren *et al.*, 2002).

ApoproteinC-II functions as an activator for lipoprotein lipase, and plays central role in the metabolism of plasma triacylglycerol. But at higher concentrations, apoC-II acts as an inhibitor of lipoprotein lipase (Pulawa *et al.*, 2007).

ApoproteinC-III also inhibits lipoprotein lipase and the concentration of plasma apoC-III correlate with hypertriacylglycerolemia (McConathy *et al.*, 1992, Shoulders *et al.*, 1991)

ApoproteinE is considered important in the metabolism of triacylglycerol-rich lipoproteins and to bind to a specific membrane receptor in liver. A genetic disease, called type III hyperlipoproteinemia, results from naturally occurring mutations in the apoE gene. The disease results in high plasma cholesterol and triacylglycerol level correlate to premature atherosclerosis, and xanthomas (Lohse *et al.*, 1991).

There are other apoproteins additions to those major apoproteins such as apoproteinD (Yang *et al*, 1994), apoproteinJ (de Silva *et al*, 1990), apoproteinM (Xu and Dahlback, 1999) which mainly associated with HDL, however their functions on lipoprotein metabolism have not yet fully known.

2.2 Lipoproteins Metabolism

2.2.1 Forward Cholesterol Transport

The dietary fatty acids (FA) are absorbed from the gut and converted to triacylglycerol which incorporated into chylomicrons, including cholesterol, phospholipids and apoB-48. Chylomicrons enter the plasma via the intestinal lymph and enter the circulation by way of the thoracic duct. Once in the blood, chylomicrons acquire apoC-II for activate lipoprotein lipase (LPL), which is most active within the capillaries of adipose tissue, cardiac muscle, skeletal muscle, and lactating mammary gland. LPL hydrolyses the triacylglycerol in chylomicrons to fatty acids, which are taken up by muscle cells for oxidation or adipocytes for storage. The remaining particles, the chylomicron remnants, are removed from the circulation by the liver through binding of their surface apoE to LDL receptor or LDL receptor related protein. The hepatocyte reassembles these chylomicron remnants along with endogenous triacylglycerol and cholesteryl esters into VLDL and secretes them into the circulation. Like chylomicrons, VLDL particles are triacylglycerol-rich and contain apoC-II and apoE but, they are smaller, contain fewer triacylglycerol and apoB-100 instead of apoB-48. As with chylomicrons, VLDL triacylglycerol are hydrolyzed by LPL which activated by apoC-II and the resultant VLDL remnants or IDL which further are taken up by liver receptors via apoE or converted to LDL. IDL triacylglycerol are hydrolyzed to free fatty acid by hepatic lipase; apoC-II is transferred to HDL while apoB-100 is reused for synthesis of LDL. LDL are the principal form in which cholesterol is transported from liver to tissues, and HDL play the primary role in returning excess cholesterol from tissues to the liver for metabolism or excretion.

2.2.2 Reverse Cholesterol Transport

The metabolic balance of cholesterol is accomplished by reverse cholesterol transport by HDL. HDL are synthesized in the intestinal mucosal cells and liver and secreted as disk-shaped nascent HDL particles containing only a small amount of

phospholipids and apoA-I (pre-β-HDL). Nascent HDL absorb free cholesterol from peripheral cells by binding to cell-surface receptors and then free cholesterol is absorbed from a “donor” cell membrane to the surface of the HDL. After that, apo A-I activate lecithin-cholesterol acyltransferase (LCAT) which catalyzes cholesterol’s esterification, the cholesteryl ester molecules are formed and move into the core of the HDL particle due to their lypophilic. Now the surface of the HDL particle has more free space for further cholesterol adsorption. With continuation of this process, HDL gradually mature to its spherical shape (α-HDL). Cholesterol from the mature HDL particles may be eliminated from the body through the liver by two pathways, a direct pathway and an indirect pathway. The direct pathway involves binding HDL to SR-B1. The indirect pathway involves transfer of cholesteryl esters from HDL to apoB–containing lipoproteins (VLDL, IDL, and LDL) mediated by cholesteryl ester transfer protein (CETP).

The overall of lipoproteins metabolism in human is showed below.

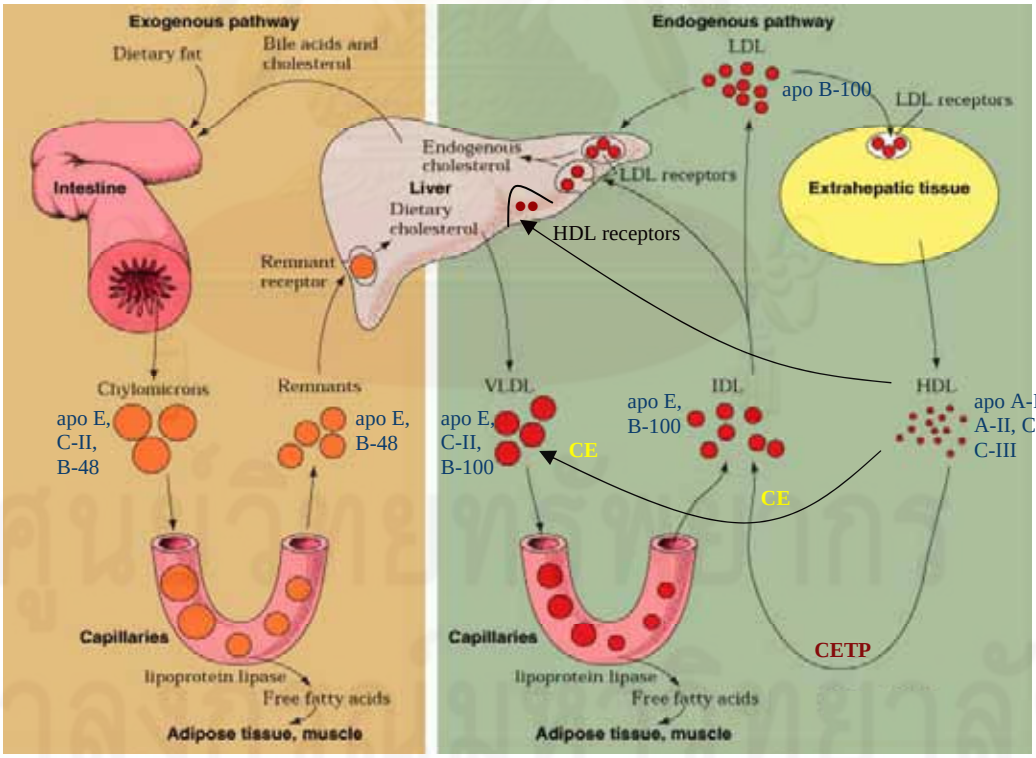


Figure 2.2 Overview of lipoproteins transports pathways in human (Adapted from Voet and Voet, 1995).

2.3 Lipoproteins and Atherosclerosis

Atherosclerosis is a disease primarily of the elastic arteries, large and medium-sized muscular arteries. The basic lesion– the **atheroma**, or fibrofatty plaque – consists of a raised focal plaque within the intima, having a core of lipid (mainly cholesterol and cholesterol esters) and a covering fibrous cap. The resultant roughening of the arterial wall promotes the formation of blood clots, which may also occlude the artery. A blood flow stoppage, known as an **infarction**, causes the death of the deprived tissues. Although atheromas can occur in many different arteries, they are most common in the coronary arteries, the arteries supplying the heart. This results in myocardial infarctions or “heart attacks”. Atherosclerosis is the most common and important form of arteriosclerosis.

Arteriosclerosis means hardening of the arteries. Arteriosclerosis marked by proliferation or hyaline thickening of the walls of small arteries and arterioles (Cotran *et al*., 1994).

The initial pathogenesis of atherogenesis has been considered by the accumulation of lipids within the artery walls, called lipid-filled foam cells. Later foam cells develop into fatty streaks (grossly flat, lipid-rich lesion consisting of both macrophages and some smooth muscle) and the fatty streak is the first signs of atherosclerosis. Later, the fibrous plaque begins as changes in the structure of the artery or fatty streaks. As atherosclerosis progresses, the streaks gradually change, becoming larger and more complex, turning into intermediate lesions. When a fibrous plaque becomes unstable and breaks or tears, thrombi can form on the surface of the plaque. If these clots are large enough, they can block the artery that has already been narrowed by the fibrous plaque (Benditt and Schwartz, 1988; Epstein, 1999; Toborek and Kaiser, 1999; Rosenfeld, 2000).

2.3.1 TRLs and Atherosclerosis

Triacylglycerol-rich lipoproteins (TRLs) compose with heterogeneous lipoproteins, chylomicrons and VLDL including their remnants. Chylomicrons are synthesized by intestine after ingestion of dietary fat. Not all of TRLs are atherogenic,

chylomicrons are too large to penetrate the endothelial cell layer, but chylomicron remnants are small enough to penetrate and to participate in atherosclerotic lesion development (Mamo *et al.*, 1998) and induce foam cell formation (Yu and Mamo, 2000). Chylomicron remnants also have been shown to impair normal endothelial function (Zheng and Liu, 2007), to be chemically modified, and to accumulate in the sub-endothelial space in the same way as LDL do (Botham, 2008).

2.3.2 LDL and Atherosclerosis

Low density lipoproteins (LDL) have been considered to relate directly to the risk of atherosclerosis. *In vitro* study demonstrates that an increase in plasma LDL levels leads to an increased rate of LDL intrusion into the artery wall consequent to theirs oxidization in endothelial cells and macrophages present in the artery wall (Schwenke *et al*., 1989).

Oxidized LDL stimulates endothelial cells to express monocyte chemotactic protein-1 (MCP-1) which attracts monocytes from blood into artery wall. The oxidized LDL then promotes differentiation of these monocytes into macrophages within the artery wall. The macrophages express scavenger receptors capable of binding and taking up the oxidized LDL. The resulting cellular accumulation of cholesterol leads to the formation of foam cells, the hallmark cell of developing atherosclerosis. The hypothesis of atherogenic mechanism is illustrated in figure 2.3.

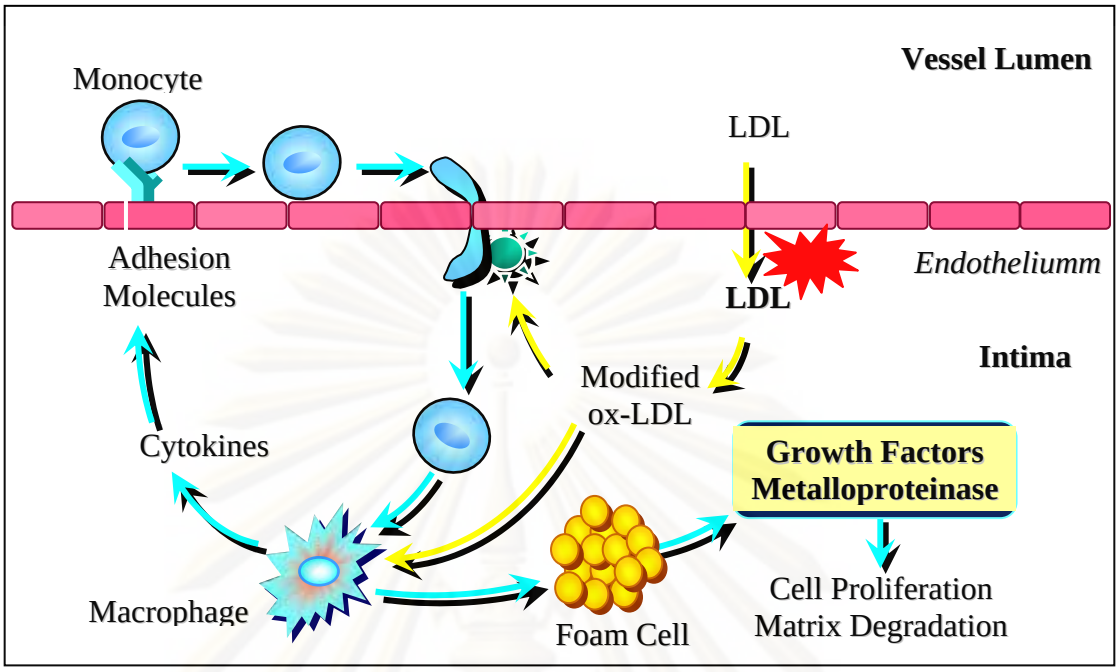


Figure 2.3 Schematic of a hypothesis of oxidative modification of LDL correspond to atherogenic mechanism (Ross, 1999).

Macrophages and foam cells secrete growth factors that lead to cell proliferation and proteinases that break down the matrix of the vessel wall. These processes lead ultimately to the development of advanced, complicated, rupture-prone atherosclerotic plaques. Macrophages in the artery wall also secrete cytokines, including tumour necrosis factor (TNF)-α and interleukin-1, that stimulate endothelial cells to express the adhesion proteins, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin. These adhesion proteins bind blood monocyted, making them accessible for recruitment into the artery wall by MCP-1, thus creating a vicious cycle that greatly amplifies the effects of the modified LDL.

It has been suggested that LDL subpopulations may vary in their atherogenic potential. Subjects with coronary heart disease (CHD) frequently have an increased concentration of small dense LDL. Increasing of atherogenic potential of small dense LDL appear to be related to a number of physicochemical and metabolic properties of these particles, including reduced LDL receptor affinity, greater propensity for

transport into the sub endothelial space, increased binding to arterial wall proteoglycans, and readily oxidized than larger and less dense LDL (de Graaf *et al.*, 1991). Although these are *in vitro* findings, they support the concept that small dense LDL may be related directly to the development of atherosclerosis.

2.3.3 HDL and Atherosclerosis

Unlike LDL, HDL is associated with a reduced risk of atherosclerosis. The mechanisms of HDL protect against atherosclerosis still uncertain, but may relate to some of their known functions such as HDL reverses the transportation of cholesterol from peripheral tissues back to the liver. In addition, *in vitro* studies show that HDL have the protective effect against the oxidative modification of LDL both by transition metal ions and by cells in tissue culture, which is dependent on time of incubation and the concentration of HDL. This is for a wide range of indices of lipid peroxidation and change in the uptake of oxidized LDL by macrophages. One of the enzymes that make HDL have the protective effect against LDL oxidation is paraoxonase (PON1). PON1 is synthesized primarily in the liver and a portion is secreted into the plasma, where it is associated with HDL. (Aviram, *et al.*, 1998; Navab, Hama, Cooke, Ananthramaiah, Chaddha, *et al.*, 2000; Navab, Hama, Cooke, Ananthramaiah, Hassan, *et al.*, 2000).

In addition to anti-oxidant property, HDL also have anti-inflammatory and anti-thrombotic properties. The anti-inflammatory property, HDL inhibits the expression of the adhesion proteins, VCAM-1, ICAM-1, and E-selectin, in endothelial cells. These adhesion proteins are fundamental to the recruitment of monocytes into the artery wall in the early stage of atherosclerosis. For the anti-thrombotic property, HDL stimulate the generation of nitric oxide which reduces the endothelial dysfunction that may precede the development of atherosclerosis. Moreover, recently HDL has been shown to promote the repair of damaged endothelium. Therefore, HDL has several properties that contribute to their ability to protect against the development of atherosclerosis.

2.4 Type2 Diabetes

Type2 diabetes (DM2) or formerly named as non-insulin-dependent diabetes mellitus (NIDDM) which is a result from the body's inability to respond properly to the action of insulin produced by the pancreatic beta-cells or insulin resistance. Insulin resistance develops over time in genetically predisposed individuals and also from dietary and lifestyle factors. Unlike type1 diabetes or formerly named as insulin-dependent diabetes mellitus (IDDM) which is a result of destruction of the insulin-producing pancreatic beta-cells, often due to autoimmunity, leading to an impaired insulin production (World Health Organization, 2009 : online).

Over long-term period, both forms of diabetes cause damage to the retina of the eye, the kidneys, the nerves, and the blood vessels. Diabetes accelerates atherosclerosis, (the formation of fatty plaques inside the arteries), which can lead to blockages or a clot (thrombus). Such changes can then lead to heart attack, stroke, and decreased circulation in the arms and legs.

The initiation of atherosclerosis in diabetes is the dyslipidemia especially in postprandial hypertriacylglycerolemia which lead to the decreasing of HDL cholesterol level, and predominance of small dense LDL particles.

2.4.1 Effect of Insulin Resistance on Dyslipidemia in Type2 Diabetes

Postprandial dyslipidemia on hypertriacylglycerolemia is considered as a more powerful risk factor for cardiovascular disease than fasting normaltriacylglycerolemia. From the prospective study of Bansal and college which studied the association of fasting and postprandial triacylglycerol with incident cardiovascular disease. The healthy US women (20,118 fasting and 6,391 nonfasting) participating in this study and undergoing follow-up for a median of 11.4 years. Triacylglycerol levels were measured in blood samples obtained at time of enrollment. The results showed nonfasting TAG levels were associated with incident cardiovascular events, independent of traditional cardiac risk factors, levels of other lipids, and markers of insulin resistance; by contrast, fasting TAG levels showed little

independent relationship. Nonfasting TAG levels maintained a strong independent relationship with cardiovascular events in fully adjusted models (hazard ratio, 95% CI) for increasing tertiles of levels: 1.00, 1.44 (0.90-2.29), and 1.98 (1.21-3.25) with *P*=.006 for trend. TAG levels measured 2 to 4 hours postprandially had the strongest association with cardiovascular events (fully adjusted hazard ratio (95% CI) for highest *vs.* lowest tertiles of levels, 4.48 (1.98-10.15) with $P<0.001$ for trend, and this association progressively decreased with longer periods of fasting (Bansal *et al*., 2007).

Type2 diabetic patients always have postprandial dyslipidemia especially triacylglycerol level mainly in triacylglycerol-rich lipoproteins (TRLs), although they have normal triacylglycerol level in fasting state. Rivellese and college studied the response of 7 patients with type2 diabetes mellitus with optimal fasting triacylglycerolemia (92.0±31.0 mg/dl) and optimal blood glucose control, HbA1C 6.17±0.20%, and 5 non diabetic controls (TAG 77.9±8.8 mg/dl) to a fat-rich meal. The main abnormality in postprandial lipid response of diabetic patients was large VLDL of both endogenous and exogenous origins. In these particles, apoB-48, apoB-100, cholesterol and TAG increment areas significantly higher in diabetics compared with controls (7.08±2.65 *vs.* 1.17±0.88 mg/liter.h, 65.5±11.5 *vs.* 12.4±1.77 mg/liter.h, 29.7±3.9 *vs.* 13.1±3.1 mg/dl.h, 170±31 *vs.* 94±22 mg/dl.h (all $P<0.05$; mean±SEM)) (Rivellese *et al.*, 2004).

An increased level of triacylglycerol-rich lipoproteins (TRLs) lead to an atherogenic lipoprotein phenotype characterized by a predominance of small dense LDL and a decreased level of HDL (Taskinen, 2003). These dyslipidemia have been considered to associate with an increased risk of cardiovascular disease (CVD) (Karpe, 1999). Moreover, the postprandial lipidemia is also associated with the endothelial dysfunction and generation of oxidative stress in type2 diabetic patients (Anderson *et al.*, 2001). This may explain why diabetic patients have higher risk to develop coronary heart disease and cause of death in patients with type2 diabetes.

The study by Annuzzi and college clearly showed that insulin resistance plays a vital role in development of postprandial lipid abnormalities by comparison of postprandial lipidemia between 8 type2 diabetes and 7 non-diabetic subjects who had normal fasting triacylglycerol level between 0.87±0.08 mmol/l (99.2±7.1 mg/dl) and 1.12±0.13 mmol/l (77.1±11.5 mg/dl). To evaluate the role of insulin resistance in development of the postprandial hyperlipidemia and diminish the hyperglycemia in diabetic subjects, blood glucose concentration in both groups were maintained to the same level about 7.8 mmol/l (141 mg/dl) by infuse 33% glucose solution into a cubital vein during 8 hours. The results showed only type2 diabetes with lower insulin sensitivity had higher postprandial levels of lipids and apoB in large VLDL (increment area for TAG 1814±421 *vs.* 549±153 μmol/lx6 hours or 160.7±37.3 *vs.*48.6±13.6 mg/dlx6 hours; $P<0.05$; cholesterol 694±167 *vs.* 226±41 μmol/lx6 hours or 26.84±6.5 *vs.* 8.7±1.6 mg/dlx6 hours; $P<0.05$; apoB-48 6.3±1.0 *vs.* 2.6±0.7 mg/lx6hours; NS) (Annuzzi *et al.*, 2004).

Beside of postprandial states, in fasting state insulin also play the possible role of diabetic dyslipidemia by reduce suppression of hydrolysis of stored triacylglycerol, so increase release of free fatty acids from adipose tissue and impaired insulin-mediated skeletal muscle uptake of free fatty acids lead to increase fatty acid delivery the liver and deposit in the form of triacylglycerol. Increase triacylglycerol in liver increase triacylglycerol production, leading to drive hepatic VLDL production especially large VLDL or VLDL1. These were confirmed with the study of Adiels and college. They determine the kinetics of apoB and TAG in VLDL1 and VLDL2 after a bolus injection of [$^2H_3$]leucine and [$^2H_5$]glycerol in 20 control subjects and 10 type2 diabetic subjects. The diabetes had mild dyslipidemia, with significantly higher total plasma TAG (1.85±0.42 *vs.* 1.35±0.48 mmol/L or 163.9±37.2 *vs.* 119.6±42.5 mg/dl), lower HDL (0.96±0.30 *vs.* 1.37±0.22 mmol/L or 37.1±11.6 *vs.* 53.0±8.5 mg/dl), and higher plasma glucose (176±39 *vs.* 106±10 mg/dL) than controls. The production rates of VLDL1 apoB and VLDL1 TAG were both significantly higher in type2 diabetes than in control subjects ($P<0.01$). In all subjects, the VLDL1 apoB and TG production rates were correlated significantly with plasma glucose ($P<0.001$), insulin

(apoB, *P*<0.01; TAG, *P*<0.05), and HOMA-IR (<0.001). Notably, VLDL1 TAG production was correlated with free fatty acids (FFAs) (Adiels *et al*., 2005).

In addition to the overproduction of large VLDL, insulin resistance and hyperinsulinemia also influences apoB-48-containing lipoprotein secretion and catabolism in type2 diabetes in both the fasting and postprandial states (Duez *et al.*, 2006 and Hogue *et al*., 2007). The study of Hogue and college which examine the in vivo kinetics of TRL apoB-48, VLDL, IDL and LDL apoB-100 in 11 type 2 diabetic subjects with severe hypertriglyceridemia and 13 normolipidemic non diabetic controls using a primed-constant infusion of L-[5,5,5-D_3]leucine for 12 hours in the fed state. Diabetic subjects had significantly higher fasting glucose level (157.836.0 *vs*.87.5±7.2 mg/dl, *p*<0.0001), higher fasting insulin level (87±45 *vs.* 57±14 pmol/l, *p*<0.03), higher plasma triglyceride (407.4±153.2 *vs.* 108.9±59.3mg/dl, *p*<0.0001), and lower HDL cholesterol levels than controls (33.3±6.2 *vs.* 46.0±9.3 mg/dl, *p*<0.0009). The results showed that type2 diabetes had an increasing of TRL apoB-48, VLDL apoB-100, and IDL apoB-100 production rate and reduced fractional catabolic rates of these lipoprotein subfractions. This study provides evidence that type2 diabetes also associated with both increased production and decreased catabolism of intestinally derived apoB-48-containing lipoproteins.

This mixed contribution of VLDL and chylomicrons overproduction leading to competition for the removal of apoB-48-containing TRLs, leading to the higher peak concentrations of TRLs or delayed clearance in diabetes reflects insulin resistance. Zheng and college used kinetic model to study the metabolism of postprandial apoB-48 and apoB-100 lipoproteins and showed that intestinal and hepatic triglyceride-rich lipoproteins have similar rates of clearance and participate interactively in similar metabolic pathways, with high apoB-48 production inhibiting the clearance of apoB-100 (Zheng *et al*., 2006). This also occurred in CAD patients and the study of Groot *et al.*, 1991 found the delay clearance of postprandial lipoproteins in CAD patients compared with that of controls without coronary atherosclerosis. Suggest that postprandial lipoproteins may play a role in the etiology of cardiovascular disease.

Together with the abnormality of enzymes correlate with lipoprotein metabolism in type2 diabetes such as lipoprotein lipase (LPL), hepatic lipase (HL) and cholesterol ester transfer protein (CETP) lead to further abnormality of LDL and HDL. The activity of LPL is decreased whereas HL and CETP activity is increased.

Insulin stimulates LPL activity, so the insulin resistance state associated with decreased activity of LPL. Pruneta-Deloche and college determine the LPL activity in 10 type 2 diabetes and 10 control, following the ingestion of a lipid-rich meal. Fasting LPL activity were 91.3±15.6 in controls versus 70.1±4.8 nmol NEFA/ml/h in diabetics (P<0.001). LPL activity was increased 2 hours postprandially by 190% in controls and by only 89% in diabetics, resulting in a 35% lowering of the LPL activity area under the curve in diabetics. These data show that TRL-bound LPL activity increases in the postprandial state and is strongly reduced in type 2 diabetes, contributing to postprandial hypertriglyceridemia. Resulting to reduce clearance of TRLs, VLDL and chylomicrons, contribute to both fasting and postprandial hypertriacylglycerolemia, which are the effect of insulin resistance (Pruneta-Deloche *et al.*, 2004).

CETP activity is increased according to triacylglycerol levels which are a major determinant of CETP activity. Therefore, in the presence of increased TRLs, CETP activity is increased. Increasing of CETP activity increases the exchange of triacylglycerol from TRLs to HDL and LDL, and of esterified cholesterol from HDL to TRLs. This lead to all circulating lipoproteins become enriched in triacylglycerol, in particular HDL and LDL particles, whereas VLDL and chylomicron remnant particles become relatively enriched in esterified cholesterol, and possibly more atherogenic. These clearly showed by the study of Guérin and college which investigated the potential relationship between CE transfer and the appearance of an atherogenic dense LDL profile and the degree of triglyceridemia influence the CETP-mediated redistribution of HDL CE to VLDL1 and dense LDL in 38 type2 diabetes and 14 healthy normolipidemic non diabetic volunteers (TAG<100 m/dl). Type2 diabetic subjects were divided into 3 subgroups according to fasting plasma triacylglycerol levels: levels: group 1, TAG<100 mg/dl; group 2, TAG 100-200 mg/dl; and group 3 TAG>200 mg/dl. Plasma levels of dense LDL subfractions were

strongly positively correlated with those of plasma TAG (r=50.471; P<0.0003). The rate of CE mass transfer from HDL to apoB-containing lipoproteins was significantly enhanced in highest TAG group compared with other groups. Moreover, the relative proportion of CE transferred from HDL to VLDL1 in type 2 diabetes patients increased progressively with increase in plasma TG levels (Guérin *et al.,* 2001)

HL has both triacylglycerol and phospholipid hydrolytic activities. The triacylglycerol content of HDL particles has been shown to determine which of the phospholipase or triacylglycerol lipase activities of hepatic lipase act on them. Triacylglycerol-rich HDL particles are better substrate for HL and enhance the clearance rate. In the insulin resistant hypertriacylglycerolemia state, HDL particles more likely to undergo catabolism, so HDL particle numbers and HDL-c concentrations are reduced. LDL particles that are triacylglycerol enriched due to the hypertriacylglycerolemia are also converted by the triacylglycerol lipase activity of hepatic lipase into smaller and denser particles. The correlations between HL activity and parameters of the insulin resistance (metabolic) syndrome, such as direct measures of insulin resistance and type2 diabetes, hypertriglyceridemia, and low HDL cholesterol could be found (Syvanne *et al*., 1995 and Kong *et al*., 2001). In addition, the induction of an insulin-resistant state by fructose fed Syrian golden hamster shifted the size of LDL particles from large to small and induced the Hepatocyte HL mRNA, protein mass, and postheparin plasma HL activity to increase and could be partially normalized by treatment with an insulin sensitizer (Lewis *et al.,* 2004).

The overall process of type2 diabetes dyslipidemia begin with hypertriacylglycerolemia which is the influence from insulin resistance is showed in figure 2.4. The type2 diabetes with mild to moderate hypertriacylglycerolemia may have the small dense LDL profile which is removed slowly by the LDL receptor pathway, contrast to large buoyant LDL which is cleared rapidly (Austin and Krauss, 1995). Moreover, small dense LDL particles are more easily modified by oxidation, by glycation and more atherogenic. Beside of oxidized LDL, VLDL from 12 DM2 was more susceptibility to peroxidation than 18 non diabetic subjects by the measurement of hydroperoxide levels and thiobarbituric acid-reactive substances

(TBARS) before and after *in vitro* peroxidative stress with $CuSO_4$ (Rabini *et al.*, 1999).

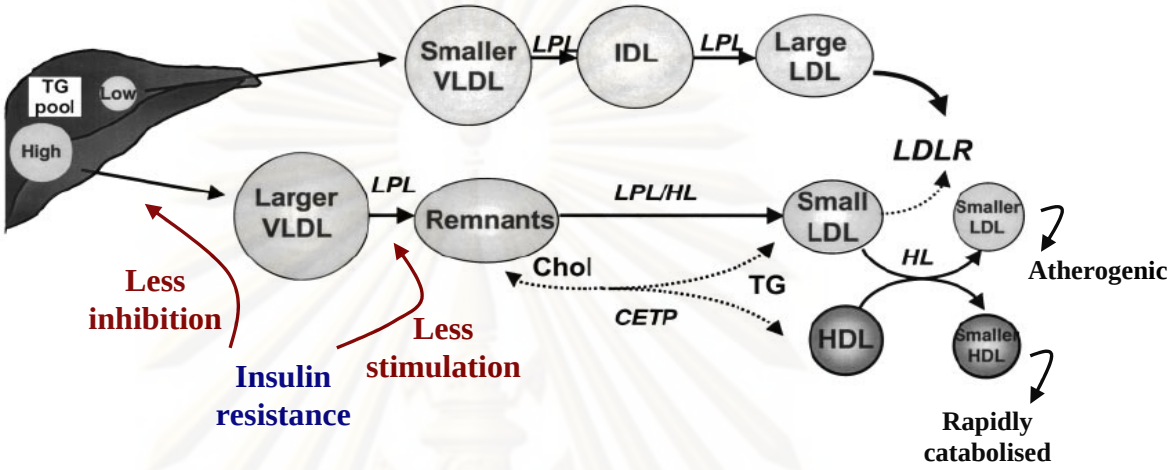


Figure 2.4 Hypothetical schemes for the relation of altered metabolism of TRLs to the development of an atherogenic lipoprotein phenotype (Adapted from Krauss, 2004).

2.4.2 Alteration of Apoproteins in Postprandial State

The alteration of apoproteins in postprandial state mainly from the apoB-48 and apoB-100 containing lipoproteins. The study in chylomicron remnants and VLDL isolated from fasting and postprandial plasma samples in ten normotriglyceridemic men. The small chylomicron remnants contained significantly more apoC-II molecules but fewer apoC-I molecules than small VLDL. Whereas the apoC-III contents of large chylomicrons decreased, the apoC-III contents of large VLDL increased postprandially (Björkegren et al., 1998). The increasing of apoC-I on postprandial chylomicrons and VLDL particles was also found in normolipidemic men without exaggerated postprandial triglyceridemia and the number of apoC-I molecules on small chylomicron remnants was strongly associated with the degree of atherosclerosis (Björkegren *et al.*, 2002). The study of Barbagallo and college which studied in hypertriacylglyceroldaemic patient, the alterations were also found in apoC-II, apoC-III and apoE which rise over time of 9 hours after meal, whereas in controls these apoproteins decreased (Barbagallo *et al.*, 1991).

Normally type2 diabetes had an increasing of apoB-48 than healthy non diabetes due to an abnormality in intestinal lipoprotein metabolism or chylomicron including its remnants in postprandial state (Curtin *et al.*, 1994). This result also found in well glycemic control type2 diabetes (HbA1C < 6.8%) although they had fasting triacylglycerol level. In addition to apoB-48, the increasing of apoB-100 resulting from the increasing of large VLDL was found in diabetes (Angela *et al.* 2004 and Rivellese *et al*., 2004). Moreover, diabetic subjects had markedly higher plasma ratios of apoCII/apoCIII and apoCIII/apo E than normal subjects which was the result of increased TRLs (Hiukka *et al*., 2005). The alterations of apoproteins mention above due to the increasing of TRLs concentration. However, postprandial apoA-V which did not have the correlation with plasma TAG concentration or with the intensity of lipoprotein lipase-dependent lipolysis elevated in diabetic patients. ApoA-V might does not play an acute or a direct role in the regulation of plasma TAG in the postprandial state.

2.5 Mass Spectrometry

Mass spectrometry can be described as the study of gas-phase ions, to determine the molecular weight and characterize the structure of a variety of molecules.

Mass spectrometer is an analytical instrument that determines the molecular weight of chemical compounds by separating molecular ions according to their mass-to-charge ratio (m/z). The result is a mass spectrum provides molecular weight or even structural information of compounds.

The first mass spectrometer was constructed in 1912 by J.J. Thomson. The development of mass spectrometer occurred after that. However, the applications of mass spectrometry were limited to small molecules. Until 1988 - 1989, two soft-ionization techniques which suitable for high molecular weight biomolecules such as proteins and peptides, were introduced that are matrix-assisted laser desorption/ionization (MALDI) (Karas and Hillenkamp, 1988) and electrospray ionization (ESI) (Fenn *et al.*, 1989).

Mass spectrometer always consists of the following basic units.

- **A sample inlet device** which introduce the compounds to ion source, e.g. a liquid chromatography or a direct insertion probe.
- **An ion source** where ions are formed from the sample.
- **An analyzer** which separates the ions according to their mass-to-charge ratio (m/z) values.
- **A detector** which count the ions emerging from the analyzer and to measure their abundance.

2.5.1 Ion Source

2.5.1.1 Electrospray Ionization (ESI)

ESI is a liquid inlet system for mass spectrometry and acts as an ionization source. ESI is a high sensitivity and easy to couple to high-performance liquid chromatography (HPLC), micro-HPLC or capillary electrophoresis.

The process of electrospray ionization (ESI) may be summarized in three major steps.

1. Production of charged droplets at the ESI capillary tip
2. Shrinkage of the charge droplets by solvent evaporation leading to very small highly charged droplets that are capable of producing gas-phase ions
3. Production of gas-phase ions from the very small and highly charged droplets.

An electrospray is produced by applying a strong electric field, under atmospheric pressure, to a liquid passing through a capillary tube with a low flow (normally 1-10 μl/min). The electric field is obtained by applying a high electric potential (typically 3-6 kV) at the end of the capillary. This field induces a charge accumulation at the liquid surface located at the end of the capillary, which will break to form highly charged droplets. A gas injected coaxially at a low flow rate allows the dispersion of the spray to be limited in space. These droplets then pass either through

a curtain of heated inert gas, most often nitrogen, or through a heated capillary to remove the last solvent molecules.

The decrease of droplet diameter via solvent evaporation process leads to an increase of the electrostatic repulsion of the charges at the surface until the droplets reach the stability limit (called the Rayleigh limit). Droplet fission occurs to produce the smaller-size stable droplet and this process is repeated (Figure 2.5).

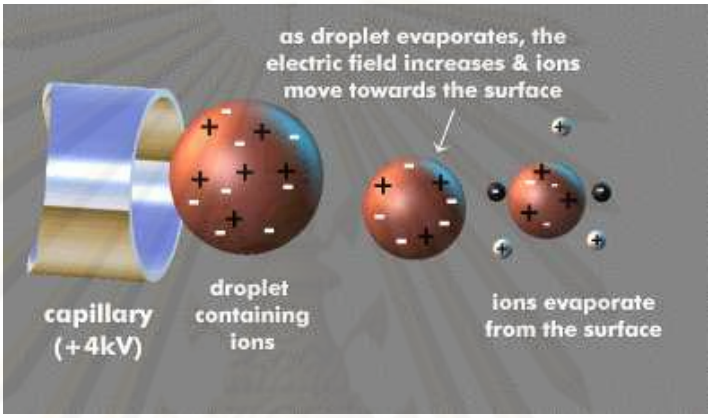


Figure 2.5 Schematic view of the electrospray ionization.

ESI is conductive to the formation of multiply charged molecules, making it possible to observe very large molecules with an instrument having a relative small mass range. How to calculate the molecular mass of the large molecules are shown below.

$$m/z = \frac{M + nH}{n}$$

where, M = molecular weight,
n = number of charges and,
H = mass of proton

Two adjacent ions in a multiply charged ion series appear at m/z of A_1 and A_2; $n_1 = n_2 + 1$, where n_1 is the number of charges on A_1 and, n_2 is the number of charges on A_2.

$$\text{Then,} \quad \frac{M + n_1H}{n_1} = A_1$$

$$\text{and,} \quad \frac{M + (n_1+1)H}{n_1+1} = A_2$$

Thus, these equations can be solved for two unknowns, M and n.

2.5.1.2 Matrix-Assisted Laser Desorption Ionization (MALDI)

MALDI is a soft ionization technique suitable for the production of intact gas-phase ions from a broad range of large, non-volatile and thermally labile compounds such as proteins, oligonucleotides, synthetic polymers and large inorganic compounds

Ions are produced by bombarding the cocrystallized mixture of matrix and sample with short pulses of UV light from a nitrogen laser. The matrixes, which provides for both desorption and ionization, absorb most of energy and transfer it to the analyte results in ionization. MALDI mainly produces the singly charged molecular species by protonation in positive ion mode. More easily deprotonated compounds are usually detected in negative ion mode. Some multiply charged ions, some multimers and very few fragments can also be observed.

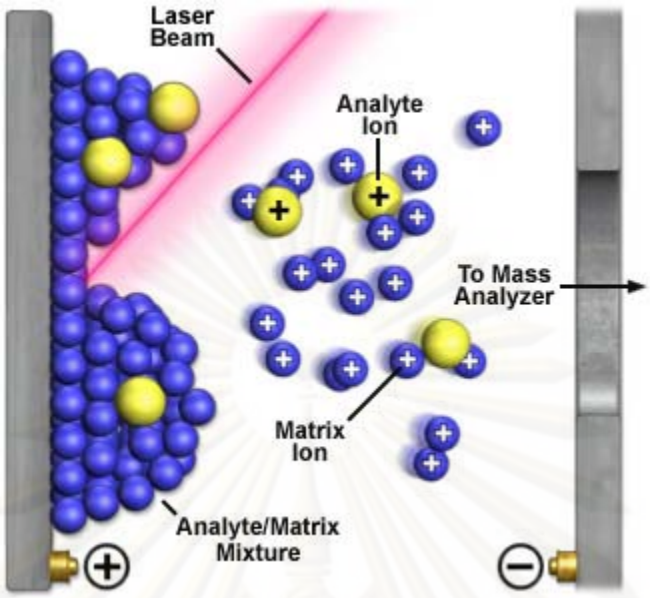
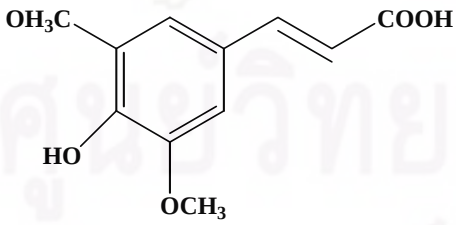
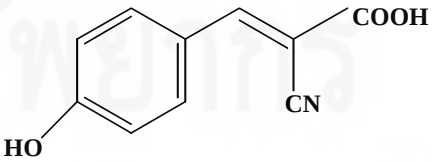


Figure 2.6 Illustrates the processes associated with matrix-assisted laser desorption/ionization (Magnet lab, University of Florida: online).

Sample preparation techniques and the use of a MALDI matrix are the crucial factor for the success of this ionization method. Typical matrices that are used for peptide and protein analysis are α-cyano-4-hydroxy-cinnamic acid (CHCA) and sinapinic acid (Kussmann *et al.*, 1997). The structures are shown below.



α-cyano-4-hydroxy-cinnamic acid (CHCA)



sinapinic acid (SA)

2.5.2 Mass Analyzers

There are many types of mass analyzer that provide different an m/z resolution and mass accuracy such as quadrupoles (Q), ion trap (IT), time of flight (ToF) and fourier transform-ion cyclotron resonance (FTICR) mass analyzers. Here, only ion traps and time of flight mass analyzers will be mentioned.

2.5.2.1 Ion Trap (IT)

The ion trap consists of a ring electrode between two endcap electrodes. Holes at the center of the endcaps allow ions to pass in and out of the trap. A high voltage RF potential is applied to the ring, while the endcaps are held at ground. The oscillating potential difference established between the ring and endcap electrodes forms a substantially quadrupolar field or a pseudo-potential well. Depending on the level of the RF voltage, the field can trap ions of a particular mass range.

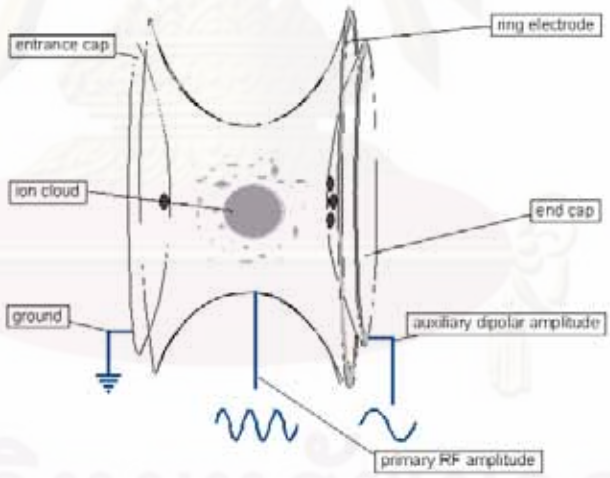


Figure 2.7 The Ion trap geometry.

Due to ions focused from an external source, they simply pass through the first endcap, roll down to pseudo-potential well, roll back up and roll out through the other endcap because of the conservation of energy. So, to extract excess energy from the ion beam, a collision gas is present in the trap and cause retention of ions in the ion trap. In practice, a trapping force from this quadrupolar field is wide enough

that the ion trap can very effectively produce full scan range of masses spectra while still offering high sensitivity.

Additional to RF voltage, an auxiliary voltage is fed to the exit endcap of the ion trap. This additional voltage is used for various purposes during the precursor ion isolation, fragmentation, and mass analysis phases of the scan sequence in tandem mass spectrometry.

Because the ion trap is a storage device, it is possible to accumulate weak signals over an extended period of time. When the ion signal is strong accumulation times may be as short as 10 µs, but increase up to approximate 1 s for infusion experiments involving trace analytes. Typical accumulation times for LC/MS and LC/MS(n) experiments range from 0.01 ms–200 ms. By varying the accumulation time, the dynamic range of the ion trap analyzer is greatly extended.

2.5.2.2 Time- of- Flight (ToF)

Time-of-flight mass analyzers often couple with MALDI. The principles base on an ion is given a fixed amount of kinetic energy by acceleration in an electric field that is generated by the application of a high voltage. Following acceleration, the ion enters a field-free region where it travels at a velocity that is inversely proportional to its m/z (from the Equation below). Because of this inverse relationship, ions with low m/z travel more rapidly than ions with high m/z. The time required for the ion to travel from the beginning to the end of the field-free flight tube is measured and used to calculate the velocity and ultimately the m/z of the ion.

Because kinetic energy (K.E.) = accelerating voltage

$$\tfrac{1}{2}mv^2 = Vz$$

$$v = ((2Vz)/m)^{1/2}$$

where, m = mass (kg)
v = velocity (m/sec)
z = charge

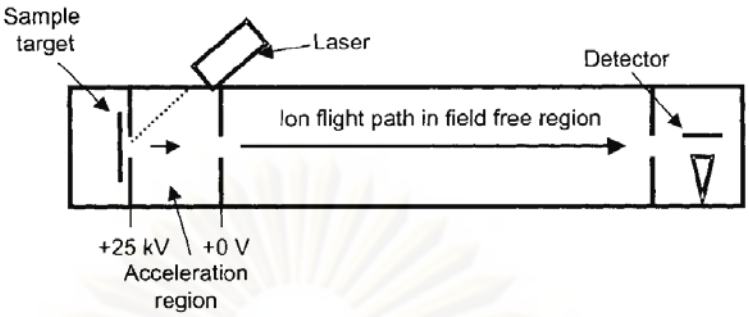


Figure 2.8 Linear time-of-flight mass analyzer.

Time-of-flight have an essentially unlimited mass range, can acquire data rapidly, and are extremely sensitive. There is no fundamental limit to the m/z range of time-of-flight that useful to analyse intact protein, unlike ion trap are limited to m/z about 3000 Th.

2.6 Tandem Mass Spectrometry

Tandem mass spectrometry means two stages of mass analysis are used in a single experiment. The first stage is used to select specific ion in a complex mixture and fragmentation in the second stage give structural information about that ion. In the case of peptide ions, the structural information is the amino acid sequence of the peptide.

Modes of scanning in tandem MS are classified in three modes.

1. Product ion scanning

Precursor ions are selected by MS1 and pass to the collision cell. Ions undergo collision with an inert gas (argon or helium) to cause dissociation and the resulting fragment (product) ions are separated in MS2.

2. Precursor ion scanning

MS2 is set to pass only the product ions of a specified m/z ratio. All ions from the ion source are scanned by MS1 and passed sequentially into the collision cell.

Those ions that fragment to give product ions of interest are revealed by the transmission of the product ions through MS2.

3. Constant neutral loss

MS1 scans the spectrum of ions from the source and MS2 scans the same mass but offset by Δm, where Δm is the difference in m/z between the precursor and product ions.

Product ion scanning mode used for determine the amino acid sequence of a peptide, precursor ion scanning or neutral losses scanning is generally used to aid in the selection of specific ions for subsequent product ion scans.

2.7 Mass Spectrometry in Proteins Research

2.7.1 Peptide Sequencing and Identification by Mass Spectrometry

Edman degradation technique continues used to protein sequencing in the field of protein biochemistry, however the major problems of this method, are time-consuming and failure to obtain amino acid sequence results from a blocked N-terminus of the protein. On comparison, mass spectrometry overcomes these problems and has more sensitivity so it becomes the current method using to peptide sequencing.

Peptide sequencing by using of product ion scanning experiment in tandem mass spectrometry based on cleavage of bonds along the peptide backbone to produce fragment ions indicative of amino acid sequence. The systematic nomenclature of sequence ions is set by Biemann (1988) who revised the nomenclature system from Roepstorff and Fohlman (1984). The a, b and c ion-type contain the original peptide N-terminus and x, y and z ion-type contain the original peptide C-terminus (Figure 2.9). A subscript number indicates the number of amino acid residues of the fragment. The obtained sequence data from MS/MS then are database searched or manually interpreted (Eng *et al.*, 1994; Yates *et al.*, 1995; Perkins *et al.*, 1999).

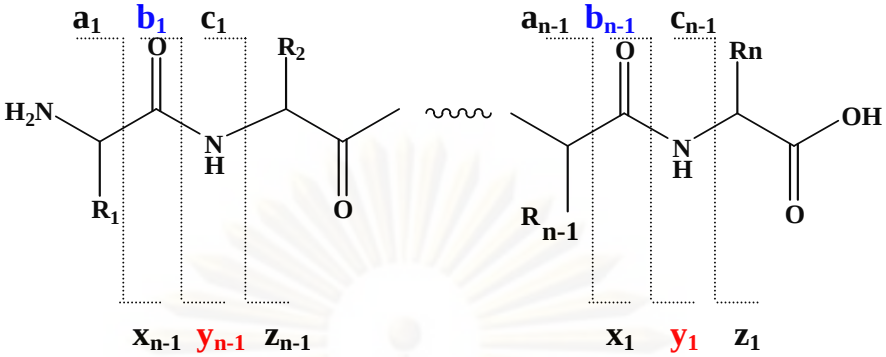


Figure 2.9 The nomenclature of the common peptide fragment ions developed by Biemann (1988).

For protein identification, mass spectrometry is used to sequence amino acid of peptide or measure mass of intact protein or peptides derived from enzymatic digestion by MALDI-ToF. Generally, trypsin is used for protein digeston. Trypsin is a specific enzyme predominantly cleaves proteins at the carboxyl side of lysine and arginine, except when either is followed by proline. The mass spectrum of peptides derived from enzymatic digestion obtain from MALDI-ToF are called the peptide mass fingerprint (PMF). The amino acid sequences results from MS/MS and PMF are used to search against database and compare to the predicted amino acid sequence of peptides from known protein. The technique originally described in 1993 consists of protein digestion, MALDI-ToF analysis and sequence database search algorithms. (Pappin *et al.*, 1993 ; Henzel *et al.*, 1993 ; Mann *et al.*, 1993 ; Yates *et al.*, 1993 ; James *et al.*, 1993).

Mass spectrometry was also used for protein modification as well such as protein glycosylation (Harazono *et al.*, 2004), phosphorylation (Ogueta *et al.*, 2000), and polymorphism (Bondarenko *et al.*, 1999). Harazono and college used electrospray ionization tandem mass spectrometry, ESI-MS/MS was used to investigate site-specific glycosylation of apoB-100. The glycopeptide ions were extracted and confirmed by the presence of carbohydratespecific fragment ions, such as *m/z* 204 (HexNAc) and 366 (HexHexNAc), in the product ion spectra. The heterogeneity of carbohydrate structures at 17 glycosylation sites was determined. The study of Ogueta

and college also successfully used tandem mass spectrometry for detec the phosphorylation size on protein. The polymorphism of protein could be identified using MALDI-ToF for determine the different molecular masses of protein polymorphism (Bondarenko *et al.*, 1999).

In clinical research, Lapolla and college used MALDI-ToF for the evaluation of the glycation level of IgG from 8 healthy subjects and also from 8 well-controlled and 14 badly-controlled type 2 diabetic patients with HbA1C 7.2±0.5 % and 10.9±2 %, respectively. They found that the masses increasing of IgG originated from non-enzymatic glycation processes. The glycation level was different among three groups of healthy and diabetic subjects, and highly correlated to HbA1C (r = 0.832, p< 0.001) (Lapolla *et al.*, 1997). Cripps and college used tandem mass spectrometry to examine paired helical filaments associated protein Tau (PHF-Tau) phophorylation which is the key of pathological hallmarks in Alzheimer disease. PHF-Tau from human Alzheimer disease brain was purified and identified the number of phosphorylation site by employing a data-dependent neutral loss algorithm to trigger MS^3 scans of phosphopeptides. This study demonstrates that qualitative and quantitative determination of PTMs across the Tau molecule can be efficiently achieved by tandem mass spectrometry in the absence of site-specific antibodies. Thus a mass spectrometric approach should make it possible to elucidate the temporal sequence of Tau PTMs in samples representing different pathological states of Alzheimer disease (Cripps *et al*., 2006).

2.8 Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE)

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is a powerful technique used to separate proteins according to their isoelectric point (*pI*) and molecular weight. This technique can separate proteins up to thousands in a single gel and developed simultaneously by Klose and O' Farrell (Klose, 1975; O' Farrell, 1975). In addition, 2D-PAGE also used to compare the different expression of protein among stage or groups and characterize the post-translational modifications of protein follow with mass spectrometry, the type- and/or the position of modifications can be identified (Sprecher, Taam and Brewer, 1984; Karlsson, 2009).

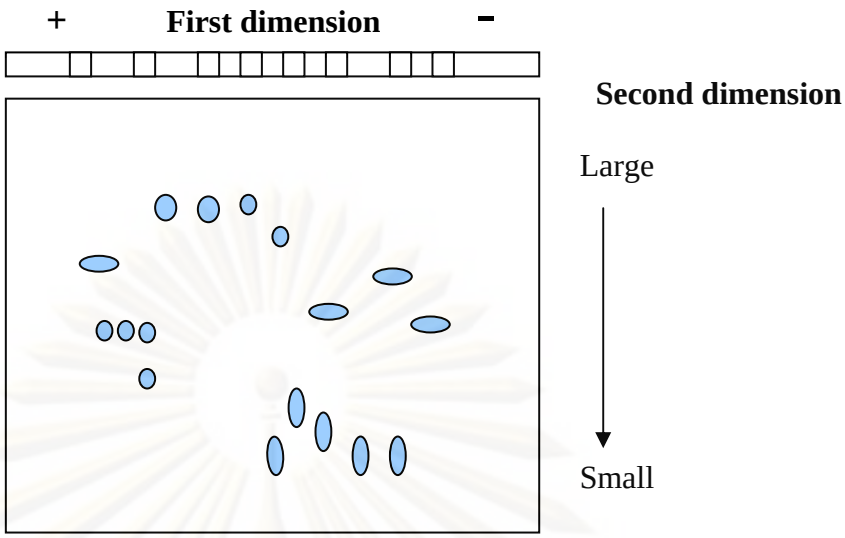


Figure 2.10 Schematic view of 2D-PAGE, separate protein according to *pI* in first dimension, and molecular weight in second dimension.

2.8.1 First Dimension

Individual proteins have individual net charge due to the differences in amino acid sequence and/or post-translational modification. The pH value which protein has zero net charge is called “isoelectric point (*pI*)”. At pH values above *pI*, protein will have a net negative charge which will make it migrate towards the anode. At pH values below *pI*, protein will have a net positive charge which will make it migrate towards the cathode. Only at pH equal to *pI*, protein will have zero net charge and no electric mobility. So, this experiment in the first dimension is called “**isoelectric focusing (IEF)**” because it involves focusing of molecules at their individual *pI* values.

When a sample solution containing proteins is loaded to an immobilized pH gradients gel (IEF gel) and electrophoresis is started, proteins migrate to their *pI* and the separation has occurred. After the IEF is completed, the IEF gel is immediately processed to the second dimension or store at -70 °C for two weeks.

2.8.2 Second Dimension

The second dimension is sodium dodecyl sulphate polyacrylamide gel electrophoresis or **SDS PAGE** used to separate proteins according to their molecular weight (MW). The smaller protein will migrate for longer distance from loading point.

SDS is added to coat protein with a uniform layer of negative charges which causes them to migrate towards the anode when placed in an electrical field. For this reason, there is a close relationship between the mobility of SDS-proteins complexes in polyacrylamide gels and the MW of the polypeptide.

Polyacrylamide gel refers to gel made from polymerized acrylamide. The uniform of pores size of gel, which restrict the movement of proteins is formed from cross link between acrylamide and N, N' methylene bisacrylamide (bisacrylamide). The pore size is inversely proportional to %T – the total percentage concentration of the monomers (acrylamide plus bisacrylamide) in grams per 100 ml.

The co-polymerization reaction of acrylamide and bisacrylamide is triggered by initiators – TEMED (tetramethylethylenediamine) and ammonium persulfate. Ammonium persulfate formed persulfate free radicals in water and activate the acrylamide monomer while TEMED serve as a catalyst to accelerate the polymerization reaction.

Although, SDS PAGE used to separate proteins according to their MW but it can only used to estimate not the exact MW.

2.9 Proteomics

In 1995, the first definition of "Proteome analysis" or Proteomics was given by Wasinger *et al.* as "The analysis of the entire protein complement expressed by a genome, or by a cell or tissue type". The proteome is a much more complex and dynamic than the genome because genomic sequence and protein function cannot be directly correlated; including proteins can be increased, decreased, co- and post translational modified. For the purpose of studying the proteome, these changes in protein amount or modifications are typically visualized by two-dimensional

polyacrylamide gel electrophoresis (2D-PAGE) (section 2.8) following by sequencing and identification of interested protein spots by mass spectrometry techniques. The overall proteomics process is showed in figure 2.11.

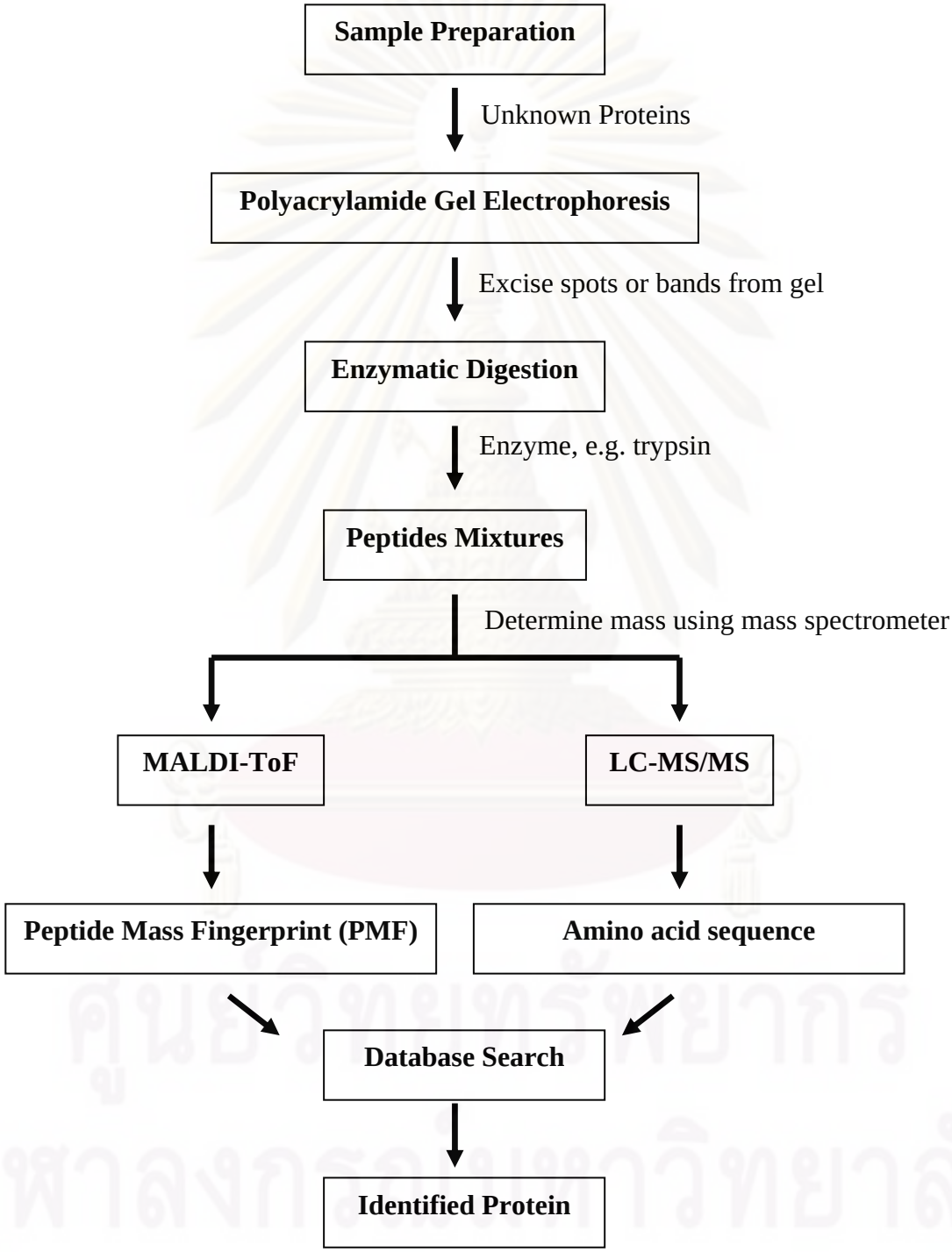


Figure 2.11 The overall process in proteomics techniques.

2.10 Proteomics Study of Lipoproteins

Recently 2D-PAGE and MS were used to study apoproteins on lipoproteins such as VLDL (Mancone *et al*., 2007), LDL and HDL in healthy human plasma (Karlsson *et al.,* 2005) and also LDL in obese subjects (Karlsson *et al.,* 2009). Furthermore, the comparative of VLDL and LDL proteome was studied by Sun *et al.,* 2010. The procedures used for proteomics study of apoproteins mainly were lipoproteins purification, delipidation, performing 2D-PAGE, staining, digestion proteins spot and protein identification by mass spectrometry.

Mancone and college studied VLDL proteins, found many apoproteins e.g., albumin, apoA-I, apoA-IV, apoE, apoCIII, apoC-IV, apoL-I, apoM, β-actin, fibrinogen-γ, prenylcysteine lyase with different isoforms and also were able identified glycosylated post-translational modification (PTM) of apoE in VLDL (Mancone *et al*., 2007). Karlsson and college studied LDL proteins, found isoform of many apoproteins in addition to apoB-100 e.g., apoC-II, apoC-III, apoE, apoA-I, apoA-IV, apoJ and apoM (Karlsson *et al.,* 2005) and they also analyzed apoproteins on LDL in obese men and found alteration of apoprotein on LDL in obese compare to non-obese subjects. They found the obese contained relative more α-1-antitrypsin, apoJ, apoC-II, acidic isoform of apoA-I than controls. On the other hand, apoA-IV and major isoform of apoA-I were significant less in LDL from the obese subjects. In addition, transthyretin was newly found in LDL (Karlsson *et al.,* 2009).

The study of HDL proteins by Karlsson and college found apoA-I, apoA-II, apoA-IV, apoC-I, apoC-II, apoC-III, apoE, apoM, serum amyloid A and serum amyloid A-IV. Alpha-1-antitrypsin and salivary alpha-amylase were identified in HDL for the first time (Karlsson *et al.,* 2005).

The comparative study of VLDL and LDL lipoproteomes by Sun and college revealed 21 associated proteins. Combined with Western blot analysis, and on the basis of the differential expression levels, proteins were classified into 3 groups: (i) VLDL>LDL: apoA-IV, apoCs, apoE, apoJ and serum amyloid A-IV; (ii) VLDL<LDL albumin, α-1-antitrypsin, apoD, apoF, apoM, and paraoxonase-1; and (iii)

VLDL=LDL: apoA-I, apoA-II, apoB-100, apoL-I and prenylcysteine oxidase-1. In addition the phosphorylation of apoA-I was detected (Sun *et al*., 2010).

From the above results showed proteomics technique provides a valuable dataset in lipoproteomes research, novel proteins could be identified including their modification and polymorphism.

CHAPTER III

EXPERIMENTAL

3.1 Experiment I: Lipoprotein profiling including LDL particles size of diabetes mellitus type2 and non-diabetic subjects.

Design of experiments

After 12 hours overnight fasting, each subject was gave a high fat meal which the total energy content of the meal is 800 Kcal/m^2 body surfaces (60% from fat, 30% from carbohydrate, and 10% from protein). The meal was ingested within 20 minutes. Only drinking water was allowed during the experiment. Blood samples before the meal and 4 hours after the meal were collected and test for blood chemistry, e.g. glucose, insulin, total cholesterol (TC), plasma triacylglycerol (TAG) and HDL-cholesterol, HbA_1C

EDTA plasma was used to separate chylomicrons, VLDL, IDL, LDL, and HDL using sequential ultracentrifugation. TC and TAG in all lipoprotein fractions were measured including the particle size of small dense LDL. Results form fasting and postprandial within and between groups were test for normal distribution with Kolmogorov-Smirnov Test before analyzed with Student's *t*-test in case within group, while between groups were analyzed with ANOVA. $P < 0.05$ was considered to be statistically significant.

Subjects

Type2 diabetes subjects

Inclusion criteria:

- Currently treated as type 2 diabetic patients
- Male or female 35-60 years old
- Available for fasting and 4 hours postprandial tests

Exclusion criteria:

- Have an evidence of acute infection, hepatic or renal disease.
- Pregnant or lactating

- HbA1C > 10%
- Fasting plasma triacylglycerol < 200 mg/dL
- Current insulin treatment
- Taking lipid lowering drugs during the previous 3 weeks

Non-diabetic subjects

Inclusion criteria:

- Healthy normalipidemic and non-diabetic subjects who have
  - Fasting plasma glucose < 110 mg/dL
  - Fasting plasma total cholesterol < 240 mg/dL
  - Fasting plasma triacylglycerol < 150 mg/dL
- Male or female 35-60 years old
- Available for fasting and 4 hours postprandial tests

Exclusion criteria:

- Have an evidence of acute infection, hepatic or renal disease.
- Pregnant or lactating

The type2 diabetic subjects were recruited from type2 diabetic patients at Theptarin hospital, whereas non-diabetic subjects were from our staff at Faculty of Allied Health Sciences, Chulalongkorn University.

After screening, the diabetic and non-diabetic subjects who have the similar body mass index, blood pressure, age and gender were divided according to insulin resistance index, which calculated from homeostatic model assessment (HOMA-IR), into subgroups of:

1. Non-diabetic subjects were categorized by HOMA-IR scores < 2.5, n = 10
2. Type2 diabetic subjects were categorized by HOMA-IR scores into 2 groups;
   - Lower insulin resistance group: HOMA-IR scores < 2.5, n = 5
   - Higher insulin resistance group: HOMA-IR scores 2.5-5.0, n = 6

The insulin resistance index is assessed by HOMA-IR (Matthews *et al*, 1985) calculated as follows:

$$\text{HOMA-IR} = \text{FIRI} \times \text{FPG}/405$$

where, FIRI is concentration of fasting immunoreactive insulin (μU/ml)
FPG is concentration of fasting plasma glucose level (mg/dl)

Written informed consent was obtained after explanation of the study protocol to all subjects. This study was approved by the Ethics Committee of Theptarin hospital and the Ethical Review Committee for Research Involving Human Subjects and/or Use of Animal in Research, Health Science Group of Faculties, Colleges and Institutes, Chulalongkorn University.

3.1.1 Materials

Serum and plasma were obtained from subjects. EDTA disodium salts, Potassium bromide (KBr), Sodium chloride (NaCl), Sucrose were purchased from Merck, Thailand. Ammonium persulfate ($(NH_4)_2S_2O_8$), Bromophenol Blue, Coomassie Blue G-250, Glycine, Tris [hydroxymethyl] aminomethane (Tris) were purchased from Amersham BioSciences, USA. Glacial Acetic acid (CH_3COOH) and Methanol (CH_3OH) were purchased from Merck, Thailand. 30% Acrylamide/Bis Solutions purchased from Bio-Rad, USA. Reagent kit for determining of total cholesterol and triacylglycerols were purchased from Human, Germany. High Molecular Weight Calibration kit for nondenaturing electrophoresis from Amersham BioSciences, USA. Amicon Ultra 100 K NMWL (Nominal Molecular Weight Limit) centrifugal filter devices from Millipore, USA.Polycarbonate clear tube and polyallomer ultralock tube 4 ml were purchased from Hitachi, Japan.

3.1.2 Methods

3.1.2.1 Preparation of Plasma from Blood

The blood plasma (EDTA 1 mg/ml blood) from subjects was centrifuged at 3,000 rpm at 4°C for 15 minutes to separate plasma and blood cells. The plasma constituted the top (yellow liquid) layer in the centrifuge tube was aspirated and further analyzed as followed.

3.1.2.2 Determination of Blood Chemistry

Total cholesterol, HDL-cholesterol, triglyceride and glucose concentrations were assayed in plasma and/or isolated lipoprotein fractions by enzymatic colorimetric methods (Human, Germany) on a humalyzer 3000 spectrometer (Human). The glycated hemoglobin (HbA_1C) was measured by immunoturbidity assay. Plasma insulin was measured by immunochemiluminometric (ICMA) assay with Immutite 100 LKIN, DPC autoanalyzer. The insulin resistance was estimated by homeostasis model assessment insulin resistance (HOMA-IR) index derived from fasting plasma insulin and glucose concentrations as previous described.

3.1.2.3 Lipoproteins Preparation and Analysis

All classes of lipoproteins were separated by sequential ultracentrifugation; the details of procedure were described later. The prepared lipoproteins were dialyzed against normal saline in Amicon Ultra 100 K NMWL (Nominal Molecular Weight Limit) (Millipore, USA) centrifugal filter devices by centrifuged at 3,000 rpm at 4°C for 30 minutes using Refrigerated centrifuge CF7D2, Hitachi with swinging bucket rotor. Total cholesterol and triacylglycerols in each fraction were determined by enzymatic reagent kits (Human, Germany).

Chylomicrons Separation

Plasma 3 ml and 1 ml of normal saline (0.85 % NaCl (w/v) in 0.1 mM EDTA) were filled in a centrifuge tube (polyallomer ultralock tube) 4 ml, placed in S100AT5 rotor and centrifuged at 21,000 rpm at 4°C for 30 minutes using Micro ultracentrifuge, Himac CS100, Hitachi. After that, the tube was cut at the clear solution beneath the chylomicrons fraction floated on the top of the tube using Tube

slicer TSU2, Hitachi. The upper fraction containing chylomicrons was collected. Transferred the infranate solution containing VLDL, IDL, LDL and HDL from the lower part of the tube to the new centrifuge tube, filled the rest of tube with normal saline and sealed.

Very Low Density Lipoproteins (VLDL) Separation

The sealed tube from above was centrifuged at 100,000 rpm at 4°C for 3 hours to separate VLDL and cut the tube as described earlier. The upper fraction containing VLDL was collected and re-centrifuged again at 100,000 rpm at 4°C for 3 hours to reduce the contaminated serum albumin. The infranant (d 1.006 g/ml) containing LDL and HDL was kept for separated LDL later.

Low Density Lipoproteins (LDL) Separation

Adjusted the density of the infranant to 1.063 g/ml by added the amount of KBr calculated from the equation

$$KBr\ (g) = \frac{V\ (D_f - D_i)}{0.7057}$$

where, V = infranate volume (ml)

D_i = initial density (1.019 g/ml)

D_f = final density (1.063 g/ml)

Mixed to dissolve the KBr, transferred the solution to the new centrifuge tube and centrifuged at 100,000 rpm at 4°C for 4 hours. The upper fraction containing LDL (d 1.063 g/ml) was collected and the infranant (d 1.063 g/ml) containing HDL was kept for separate HDL later.

LDL Subclasses Separation

Readjusted the density of LDL (d 1.063 g/ml) prepared from above to 1.050 g/ml by added 0.25 ml of H_2O to 1 ml of LDL (d 1.063 g/ml). Mixed and centrifuged at 100,000 rpm at 4°C for 4 hours. The upper fraction containing light-

LDL (l-LDL) (d 1.019-1.050 g/ml), the lower fraction containing small dense-LDL (d-LDL) (d 1.050-1.063 g/ml) was collected and further analyzed for particles size.

High Density Lipoproteins (HDL) Separation

Adjusted the density of the infranant from 1.063 g/ml to 1.210 g/ml by added the amount of KBr calculated from the equation

$$KBr\ (g) = \frac{V\ (D_f - D_i)}{0.6438}$$

where, V = infranate volume (ml)

D_i = initial density (1.063 g/ml)

D_f = final density (1.210 g/ml)

Mixed to dissolve the KBr, transferred the solution to the new centrifuge tube and centrifuged at 100,000 rpm at 4 ^{o}C for 4 hours. The upper fraction containing HDL (d 1.210 g/ml) was collected. The infranant was discarded.

3.1.2.4 Determination the Particles Size of Small Dense LDL

The particles size of d-LDL was estimated in duplicate by gradient 3-12 %T non-denaturing polyacrylamide gel electrophoresis with stacking gel using a modified method of Nichols *et al*, 1986. The gradient gel was prepared using a two-chamber self-constructed gradient former connected with peristaltic pump. Light solution (3% T) and heavy solution (12 %T) were prepared and filled in separate chamber.

The particle size calibrator for determine the particle size of d-LDL composed with a standard proteins mixture (High Molecular Weight Calibration kit for nondenaturing electrophoresis, Amersham BioSciences, USA) and standard LDL (d 1.050-1.063 g/ml).

The standard proteins mixture contained the following proteins: Thyroglobulin, diameter 17.0 nm, Ferritin, diameter 12.2 nm, lactate dehydrogenase, diameter 8.1 nm.

Standard LDL (std-LDL) for determination of d-LDL particles size was prepared using ultracentrifugation method as described above. Std-LDL was dialyzed against normal saline in Amicon Ultra 100 K NMWL device and further examined using Transmission Electron Microscope (TEM) in negative staining, which prepared by mixed a small aliquots of std-LDL (50-80 ug/ml) with an equal volume of 2% sodium phosphotungstate (PTA), pH 7.4 and placed a droplet on a Formvar/carbon-coated grid, left for 3 minutes. The excess fluid was removed with filter paper and dried at room temperature (Figure 3.1.) before examined in the JEOL, JEM-2100 TEM at The Scientific and Technology Research Equipment Centre, Chulalongkorn University.

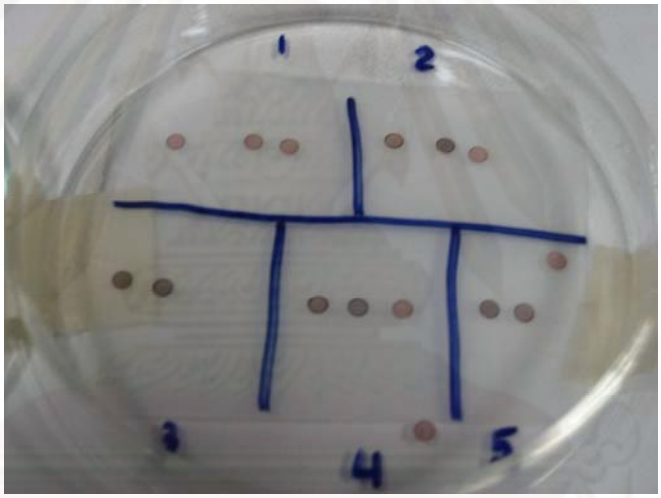


Figure 3.1 The formvar/carbon-coated grid containing standard LDL and ready to examine in transmission electron microscope.

The standard proteins mixture (Amersham BioSciences, USA) was reconstituted with 100 µl of electrophoresis buffer (Tris-glycine, pH 8.3) per vial and applied 10 µl per well. For std LDL and sample were prepared by mixed an equal volume of std LDL or sample with sample buffer prepared from 10 µl of 1% Bromophenol blue solution, 300 µl of sucrose solution (40% w/v) and applied 15 µl per well.

Electrophoresis was run at 4 °C, under the conditions; prerun at 125 V: 20 minutes, run at 70 V: 25 minutes and 175 V: 16 hours using SE-260 mini-vertical gel elecrtrophoresis unit (Amersham BioSciences, USA). The electrophoresis buffer contained 192 mM glycine and 25 mM Tris-HCl, pH 8.3.

After electrophoresis finished, gels were stained with Coomassie Blue Staining (10% acetic acid, 40% methanol, 0.1% Coomassie blue G250) for 30 minutes. Destained with destain solution (7% acetic acid, 12% methanol) until achieved suitable background.

3.1.2.5 Statistical Analysis

All variables were normal distributions tested by One Sample-Kolmogorov-Smirnov Test. The statistical analysis was performed by SPSS Statistics Base version 17.0 (for Chulalongkorn University by SPSS (Thailand) Co., Ltd.) Continuous data was expressed as mean ± SEM. Statistical data was analyzed with paired-samples *t*-test for compare the differences between fasting and postprandial data. Differences among 3 groups were performed by analysis of variance (ANOVA) with Duncan's New Multiple Range Test. The correlations between some variables were performed with Pearson test. $P < 0.05$ was considered to be statistically significant in all statistical analysis.

3.2 Experiment II: Study on the influence of insulin resistance on the alteration of apolipoprotein of very low density lipoprotein (VLDL) between diabetes mellitus type2 and non-diabetic subjects in postprandial state.

Design of experiments

EDTA plasma from 12 hours fasting and 4 hours postprandial from diabetic and non-diabetic subjects were pooled as six pools; fasting controls, fasting LIR, fasting HIR, postprandial controls, postprandial LIR and postprandial HIR. VLDL from each pools were separate using sequential ultracentrifugation VLDL and delipidated, the resulting apoproteins further analyzed with two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) in triplicate and identified with mass spectrometry both from tandem mass spectrometry (MS/MS) and peptide mass fingerprinting (PMF).

3.2.1 Materials

Serum and plasma were obtained from subjects. Ammonium bicarbonate (NH_4NCO_3), Enolase (from baker's yeast, *S. cerevisiae*), Thiourea (NH_2CSNH_2), Trypsin (Proteomics grade) were purchased from Sigma-Aldrich, Singapore. Agarose, Ammonium persulfate ($(NH_4)_2S_2O_8$), Bromophenol Blue, (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate (CHAPS), Coomassie Blue G-250, Dithiothreitol (DTT), Dry Strip Cover Fluid, Glycerol 87% (w/w), Glycine, Iodoacetamide, IPG buffer and Immobiline drystrip pH 3-10 NL, Sodium Dodecyl Sulfate (SDS), Tris [hydroxymethyl] aminomethane (Tris), Ureas (NH_2CONH_2) were purchased from Amersham BioSciences, USA. Acetonitrile, Ammonium sulfate ($(NH_4)_2SO_4$), Diethyl ether, EDTA disodium salt, Ethanol (C_2H_5OH), Formic acid (HCOOH) 98-100%, Glacial Acetic acid (CH_3COOH), Hydrochloric acid (HCl), Methanol (CH_3OH), ortho-Phosphoric acid (H_3PO_4) 85%, Sodium chloride (NaCl) were purchased from Merck, Thailand. 30% Acrylamide/Bis Solutions and Bio-Rad Protein Assay were purchased from Bio-Rad, USA.

3.2.2 Methods

3.2.2.1 Very Low Density Lipoprotein (VLDL) Preparation

VLDL were separated from fasting and postprandial plasma using sequential ultracentrifugation as described in section 3.2.3 and the second step of ultracentrifugation at the same condition was performed to reduce the contamination of albumin before further delipidated in the next step.

3.2.2.2 Delipidation of Lipoproteins

VLDL was delipidated in methanol-diethyl ether solvent system according to Karpe's method (Karpe *et al*, 1994) with slightly modified. Briefly, 750 µl of a lipoproteins fraction was injected into 4 ml of iced-cold methanol in a round bottom glass tube followed by 4 ml of iced-cold diethyl ether, and centrifuged at 3,000 rpm at 4°C for 30 minutes using Refrigerated centrifuge CF7D2, Hitachi. The solvent was aspirated and discarded; the apoproteins at the bottom was washed again with 4 ml of iced-cold diethyl ether. The resulting apoproteins were suspended in rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 0.5% IPG buffer, 18 mM DTT, 0.002% bromophenol blue) and blew with N_2 to evaporate the remaining solvent from the delipidation.

3.2.2.3 Protein Quantification

The resuspended apoproteins in rehydration buffer for IEF 1 part was diluted with 4 parts of deionized water before determined concentration of protein according to the principle of Bradford (Bradford, 1976) using the Bradford assay kit (Bio-Rad, USA). The procedure was according to the instructions of the kit.

Dye Reagent Concentrate 1 part was diluted with 4 parts of deionized water and filtered to remove particulates before used. Standard was prepared as four concentrations by serial dilution of bovine gamma globulin (1.32 g/ml, w/v). Pipetted 10 µl of each standard and sample into microtiter plate wells in duplicate and followed with 200 µl of diluted dye reagent. Mixed and incubated at room temperature for at least 5 minutes but no more than 1 hour and measured absorbance

at 595 nm using BioTek-Power Wave Microtiter plate reader (Biotek Instruments, USA). The protein concentration of sample was estimated from standard curve.

3.2.2.4 Two-Dimensional Gel Electrophoresis (2-DE)

First dimension (Isoelectric Focusing, IEF)

First dimension electrophoresis was performed using IPGphorII^{TM} (Amersham BioSciences, USA) in triplicates for each sample. Samples in rehydration buffer containing 120 μg protein per 125 μl was applied for in-gel rehydration for 12 hrs using low voltage 30 V in pH 3-10, non linear IPG strip, 7 cm and then was focused according to the instruction with slight modification. The focusing step was started at 500 V: 30 minutes, 1000 V: 30 minutes, 5000 V: 7500 Vhr and 5000 V: 15 minutes. After complete focusing the strip was used immediately for the second dimensional analysis.

Second dimension (SDS-PAGE)

Before the second dimension was performed, the focused strip need two steps of equilibration in equilibration solution containing 2% SDS, 50 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 0.002% bromophenol blue. First equilibration step, DTT was added to the equilibration solution (final DTT concentration is 1% w/v). Second equilibration step, iodoacetamide was added to the equilibration solution (final concentration is 2.5% w/v). The focused strip was equilibrated for 15 minutes in each step. After that, The equilibrated strip was placed on a 13 %T, 2.6 %C homogenous SDS-PAGE 0.1 x 16 x 16 cm gel size using the PROTEAN® II xi cell (Bio-Rad, USA) sealed with melting agarose and run with 30-50 mA until the dye front reach 1 cm from bottom.

3.2.2.5 Staining and Image Analysis

The proteins in gel from 2-DE were detected by Colloidal Coomassie Blue staining. First, gel was fixed with fixative solution (10% acetic acid, 40% methanol) at least 1 hour and replaced with water for 10 minutes, at least 3 times. Later, gel was stained overnight in working dye solution prepared by mixed four parts of dye stock solution (10% ammonium sulphate, 1.2% phosphoric acid, 0.1%

Coomassie blue G250) with one part of methanol and destained with 45-55°C water. Proteins were visualized using ImageScanner II in combination with a computerized imaging, ImageMaster^{TM} 2D Platinum software (Amersham BioSciences, USA) was used to analyze the results.

3.2.2.6 Digestion of Protein Spots

The protein spots were excised from stained gel, and placed into 0.5 ml microcentrifuge tube. The spot from protein free region was also cut to use as a control digestion. The gel plaques were washed with 100 µl of 25 mM NH_4HCO_3/50% acetonitrile and repeated until the plaques are clear. Added 100 µl of acetonitrile, followed with 25 mM NH_4HCO_3 the solution was discarded. Added acetronitrile again and the gel plaques were left to completely dry at room temperature. Trypsin (40 ng/ul) was added to each plaque and 30 µl of 25 mM NH_4HCO_3 was added to immerse the spots, incubated at 37º C, overnight. The supernatant was transferred to a separate tube and dried in vacuum centrifuge DNA110 Speed Vac (Thermo Savant).

3.2.2.7 Mass Spectrometry

Electrospray Ionization (ESI)

The tryptic digest was analyzed with HPLC (Agilent 1100 Series, USA) directly connected to ESI ion trap mass spectrometer (Esquire HCT, Bruker Daltonics, Germany). For HPLC, BioBasic C18 column, 1.0 x 150 mm (Thermo Finnigan) was used and eluted at 0.1 ml/min using a gradient of solvent A (0.1% v/v FA in 98% H_2O) and solvent B (95% ACN containing 0.1% v/v FA). A linear gradient started from 5% B to 65% B in 65 min and then from 65% B to 80% B in 5 minutes, followed by 25 minutes hold at 5% B, column temperature was set at 35 °C. The injection volume was 5 µl.

ESI mass spectrometer, the electrospray capillary potential was 3500 V relative to the end plate, the temperature of dry gas was 300°C, the dry gas and nebulizing gas were set at 8 ml/min and 35 psi respectively. Data were acquired in

positive mode. Maximum accumulation time was 200 milliseconds. For conventional mass spectra, mass range scan was set from 300 to 2500 m/z and resolution was set to give a peak width at half height of 0.4 m/z units for the monoisotopic peak of a singly charged ion. For tandem experiment on product ion scanning mode, resolution was set to give a peak width at half height of 0.6 m/z units and five precursor ions didn't belong to singly charged ions were selected automatically. Hystar coupled with Esquire Control software (Bruker Daltonics, Germany) were used for instruments control. DataAnalysis version 3.2 and Biotools version 3.1 were used for data acquisition and analysis.

Figure 3.2 HPLC directly connected with ESI-IT mass spectrometer.

Matrix-Assisted Laser/Desorption Ionization (MALDI)

The tryptic digest 0.5 µl was mix on plate with 0.5 µl of α-cyano-4-hydroxycinnamic acid, CHCA (10 mg CHCA in 1 ml of 0.1 % TFA, 50% ACN) and analyzed with MALDI-Tof (Bruker Daltonics, Germany). Data were acquired in positive reflector mode. Mass spectra were obtained from at least 50 laser shots in mass range from 500 to 3000 m/z. FlexAnalysis version 3.0 and Biotools version 3.1 were used for data acquisition and analysis.

3.2.2.8 Database search

The Results from MALDI and LC-MS/MS were submitted to database search using MASCOT search engines - www.matrixscience.com against SwissProt database. Restrictions are maximum one missed cleavage by trypsin, and cysteine modification by carbamidomethylation. All peptide mass values were considered as monoisotopic (Figure 13). Results from MALDI was search as Peptide Mass Fingerprint, MASCOT scores greater than 61 was considered significant ($p<0.05$). For tandem MS analysis, all peaks with S/N greater than 5 were search against database as MS/MS Ion Search. The peaks were identified as non-protein by database search from PMF and tandem MS were manually interpreted and confirmed by Biotools version 3.1 (Bruker Daltonics).

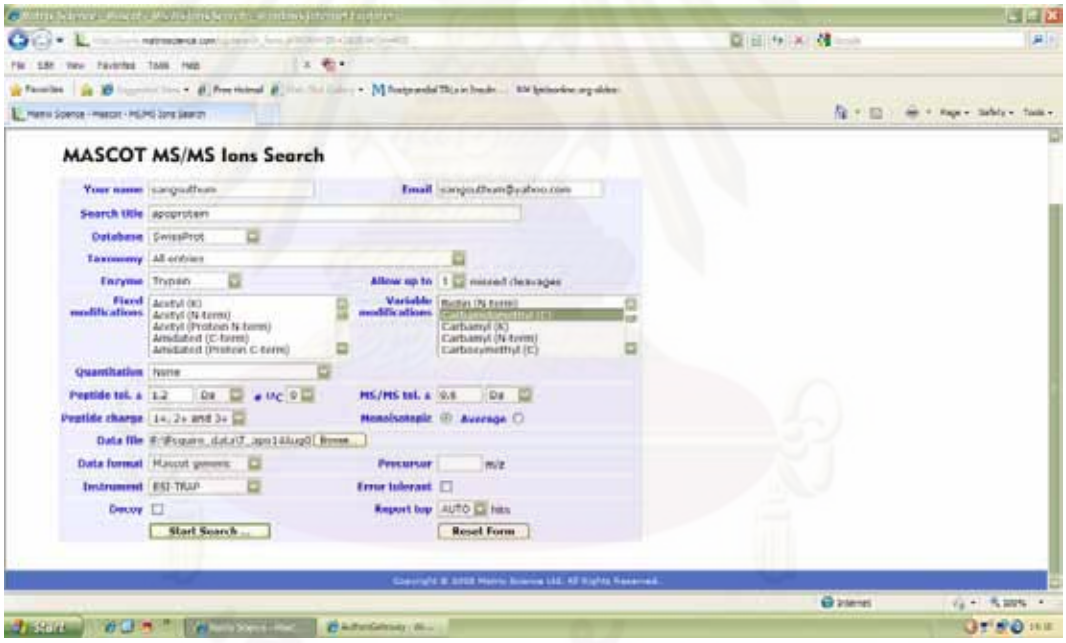


Figure 3.3 The search parameters used in MASCOT MS/MS ions search.

CHAPTERS IV

RESULTS

4.1 Experiment I: Study on the influence of insulin resistance on diabetic lipoprotein profiling including small dense LDL particles size.

4.1.1 Baseline Parameter

In this study, eleven subjects with type2 diabetic mellitus; six subjects were classified as higher insulin resistance DM2, HIR (HOMA-IR 2.5-5.0), while five subjects were lower insulin resistance DM2, LIR (HOMA-IR <2.5). The control subjects were ten non-diabetic subjects and have no history or symptoms of any known disease. All three groups were compared for age, duration of diabetes, body mass index (BMI), blood pressure, waist-to-hip circumference ratio (Table 4.1).

Table 4.1 Baseline characteristics of control and type2 diabetes subjects.

Parameters	Control (n=10)	Type2 Diabetes	
		HOMA-IR <2.5 (n=5)	HOMA-IR 2.5-5.0 (n=6)
Age, years	46.0±1.7 [a]	47.2±1.9 [a]	49.3±3.9 [a]
Duration of diabetes, years	-	7.4±2.1 [a]	5.7±1.0 [a]
BMI, kg/m^2	23.6±0.7 [a]	22.6±1.9 [a]	28.6±1.8 [b]
Blood pressure, mmHg			
-Systolic	121.0±4.8 [a]	124.6±12.9 [a]	123.2±7.7 [a]
-Diastolic	68.1±1.8 [a]	79.4±7.4 [a]	76.7±3.3 [a]
Waist-to-hip ratio	0.83±0.02 [a]	0.84±0.04 [a]	0.87±0.02 [a]

- Values are means ± SEM.
- Significant differences (p<0.05) are indicated by superscriptive alphabet in the same row, sharing the same alphabet means no significance.

The characteristics of all subjects by age, blood pressure and waist-to-hip ratio were comparable and not considered as different significantly at $p < 0.05$ including the duration of diabetes in both group. However, BMI of HIR group was different significantly from control and LIR group at $p < 0.05$ and HIR group was considered as overweight with BMI more than 25 kg/m^2.

4.1.2 Blood Chemistry Parameters between Fasting and Postprandial State in Whole Plasma

After the digestion of a lipid-rich meal, the clinical data and blood chemistry parameters from DM2 subjects varied with insulin resistance; LIR (HOMA-IR<2.5), HIR (HOMA-IR 2.5-5.0) and control were compared to that before the meal were shown in Table 4.2.

Fasting state, blood glucose in both diabetic groups (LIR 134.4±16.6 mg/dl, HIR 126.5±10.7 mg/dl) were higher than control group (89.0±2.7 mg/dl) as expected, and also HbA_1C (LIR 7.3±0.8 %, HIR 7.3±0.5 % and control 5.7±0.2 %) with significant differences (p<0.05) in control and both diabetic groups but not significantly within diabetic groups. Insulin level in fasting state of control and HIR group were significant differences at p<0.05, but were not different from LIR group, control group had insulin 5.2±0.8 μU/ml, LIR had 2.8±0.4 μU/ml and HIR had 11.5±1.2 μU/ml. HOMA-IR of control group and LIR were similar (1.2±0.2 and 1.0±0.2, respectively) whereas HIR was higher 3.5±0.4 with significant differences (p<0.05). Triacylglycerol in HIR diabetic group (147.0±12.8 mg/dl) was higher than the other groups; LIR (66.0±8.2 mg/dl), control (99.6±11.9 mg/dl), however there were not significant differences at p<0.05. Fasting LDL cholesterol were not considered to statistically differences (p<0.05) between three groups. HDL cholesterol in control (51.7±3.6 mg/dl) and LIR (51.2±3.8 mg/dl) were higher than HIR (41.7±3.1 mg/dl) with significant differences (p<0.05) between HIR and other groups but not significantly in control group and LIR.

Table 4.2 Fasting and 4 hours postprandial blood chemistry parameters in whole plasma of diabetic subjects *vs*. control.

Parameters	Control (n=10)	Type 2 Diabetes	
		HOMA-IR <2.5 (n=5)	HOMA-IR 2.5-5.0 (n=6)
Fasting			
Total cholesterol, mg/dl	203.5±13.6 [a]	192.2±14.3 [a]	185.8±6.3 [a]
Triacylglycerol, mg/dl	99.6±11.9 [a]	66.0±8.2 [a]	147.0±12.8 [a]
HDL cholesterol, mg/dl	51.7±3.6 [a]	51.2±3.8 [a]	41.7±3.1 [b]
LDL cholesterol, mg/dl	131.8±12.6 [a]	127.8±14.5 [a]	114.8±6.1 [a]
Glucose, mg/dl	89.0±2.7 [a]	134.4±16.6 [b]	126.5±10.7 [b]
Insulin, μU/ml	5.2±0.8 [a]	2.8±0.4 [a]	11.5±1.2 [b]
HOMA-IR	1.2±0.2 [a]	1.0±0.2 [a]	3.6±0.4 [b]
HbA_1C (%)	5.7±0.2 [a]	7.4±0.8 [b]	7.3±0.5 [b]
4 Hours Postprandial			
Total cholesterol, mg/dl	204.1±14.7 [a]	198.8±14.5 [a]	198.3±10.8 [a]
Triacylglycerol, mg/dl	239.2±32.6 [a]	164.6±34.2 [a]	358.0±31.3 [b]
HDL cholesterol, mg/dl	51.7±3.5 [a]	51.2±3.3 [a]	41.3±2.3 [b]
Glucose, mg/dl	99.5±2.7 [a]	135.8±16.6 [b]	129.8±10.7 [b]
Insulin, μU/ml	9.4±1.8 [a]	8.2±1.0 [a]	27.0±3.8 [b]

- Values are means ± SEM.
- Significant differences ($p<0.05$) are indicated by superscriptive alphabet in the same row, sharing the same alphabet means no significance.

After the meal, blood glucose in both diabetic groups (LIR 135.8±16.6 mg/dl, HIR 129.8±10.7 mg/dl) were still higher than control group (99.5±2.7 mg/dl) with significant differences ($p<0.05$) between control and both diabetic groups but not significantly within diabetic groups. All three groups had the increasing of insulin level with significant differences at $p<0.05$ between HIR (27.0±3.8 μU/ml), LIR

(8.2±1.0 μU/ml) and control (9.4±1.8 μU/ml). Triacylglycerol concentrations in all groups were higher than before the meal. However, only HIR was considered as statistically difference from other groups at $p<0.05$. Total cholesterol only increased in both groups of diabetes with significant differences at $p<0.05$ between diabetic groups and control, whereas HDL cholesterol level almost didn't change in all groups (control 51.7±3.6 and 51.7±3.5 mg/dL; LIR 51.2±3.8 and 51.2±3.3 mg/dl; HIR 41.3±3.1 and 41.3±2.3 mg/dl).

Table 4.3 Comparative of blood chemistry parameters from fasting and 4 hours postprandial plasma within control and diabetic subjects.

Parameters	**Control (n=10)**	**Type 2 Diabetes**	
		HOMA-IR <2.5 (n=5)	**HOMA-IR 2.5-5.0 (n=6)**
Total cholesterol, mg/dl			
-fasting	203.5±13.6	192.2±14.3	185.8±6.3
-postprandial	204.1±14.7	198.8±14.5	198.3±10.8
Triacylglycerol, mg/dl			
-fasting	99.6±11.9 *	66.0±8.2 *	147.0±12.8 *
-postprandial	239.2±32.6	164.6±34.2	358.0±31.3
HDL cholesterol, mg/dl			
-fasting	51.7±3.6	51.2±3.8	41.7±3.1
-postprandial	51.7±3.5	51.2±3.3	41.3±2.3
Glucose, mg/dl			
-fasting	89.0±2.7 *	134.4±16.6	126.5±10.7
-postprandial	99.5±3.9	135.8±16.6	129.8±13.8
Insulin, μU/ml			
-fasting	5.2±0.8 *	2.8±0.4 *	11.5±1.2 *
-postprandial	9.4±1.8	8.2±1.0	27.0±3.8
Insulin increasing, %	96.3±39.7	225±72.4 $	187.3±32.6 $

Values are means ± SEM.

* $P<0.05$ Compare parameters between postprandial and fasting state.

$ $P<0.05$ compare with control.

From table 4.3, blood chemistry parameters within group from fasting and postprandial state were compared. All three groups had the increasing of insulin level in postprandial state with significant differences at $p<0.05$; control (5.2± 0.8 and 9.4±1.8 μU/ml), LIR (2.8±0.4 and 8.2±1.0 μU/ml) and HIR (11.5±1.2 and 27.0±3.8 μU/ml) and the increasing of insulin level in percent between LIR and HIR groups was not significant differences (225±72 and 187.3±32.6%), whereas control and LIR had significant differences at $p<0.05$ (96.3±39.7 and 225±72%), control and HIR had significant differences at $p<0.05$ (96.3±39.7 and 187.3±32.6%). Triacylglycerol concentrations were higher than before the meal in all groups with significant differences at $p<0.05$; control (99.6± 11.9 and 239.2±32.6 mg/dl), LIR (66.0±8.2 and 164.6±34.2 mg/dl) and HIR (147.0±12.8 and 358.0±31.3 mg/dl). Only control group had the increasing of glucose level in postprandial state with significant differences at $p<0.05$ from fasting state (99.5±3.9 and 89.0±2.7 mg/dl).

4.1.3 The Influence of Insulin Resistance on Diabetic Plasma Triacyglycerol

To find the influence of insulin resistance on plasma triacylglycerol level, the correlation of HOMA-IR and plasma triacylglycerol both fasting and after a high fat meal were analyzed. The results show the correlation between HOMA index and postprandial triacylglycerol also HOMA index and fasting triacylglycerol were significantly correlated in diabetic subjects as shown in figure 4.1 and figure 4.2. However, these correlations were not found in non diabetic subjects.

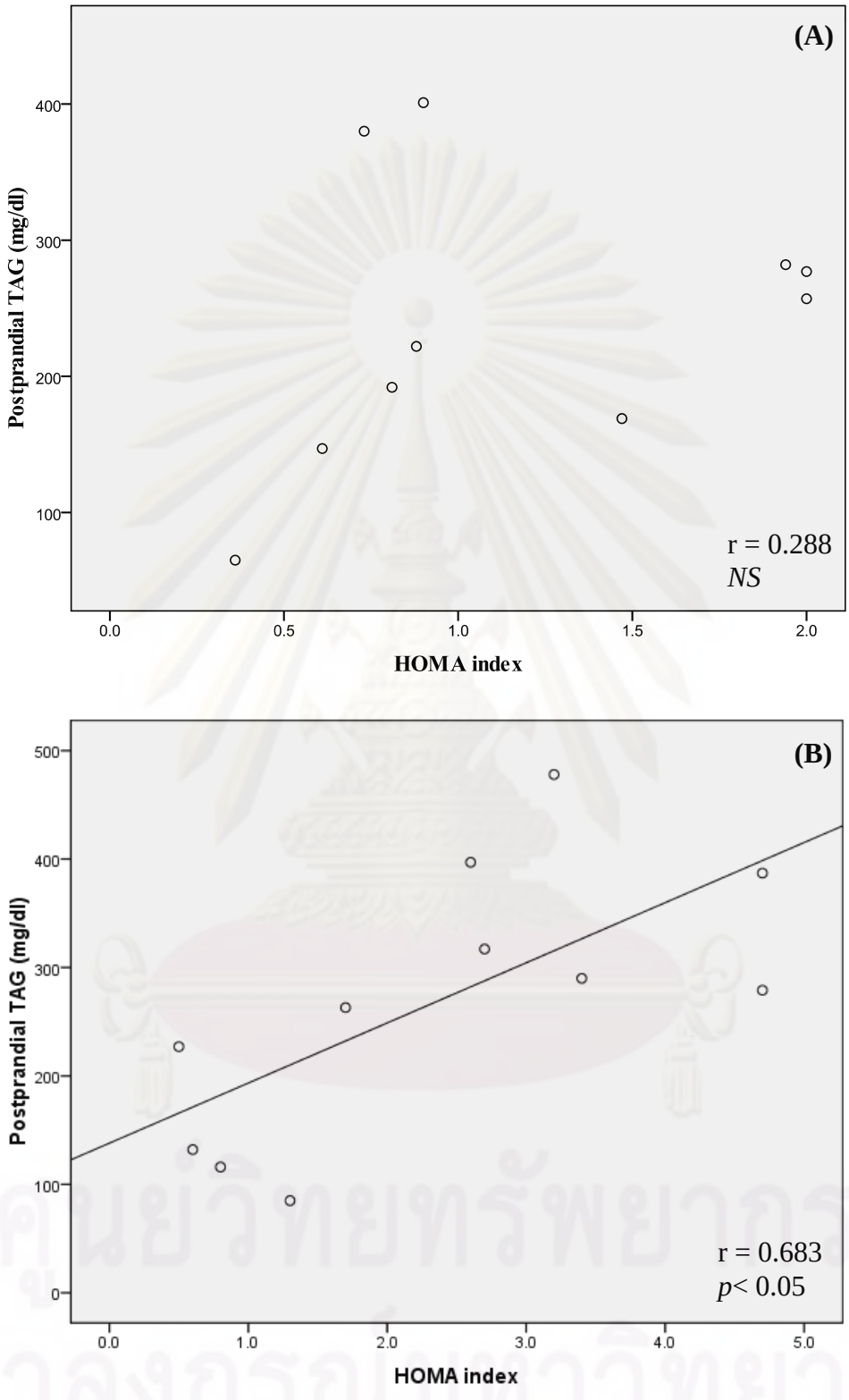


Figure 4.1 Relationship between HOMA-IR and postprandial triacylglycerol level measured 4 hour after meal in (A) control subjects and (B) type2 diabetes subjects.

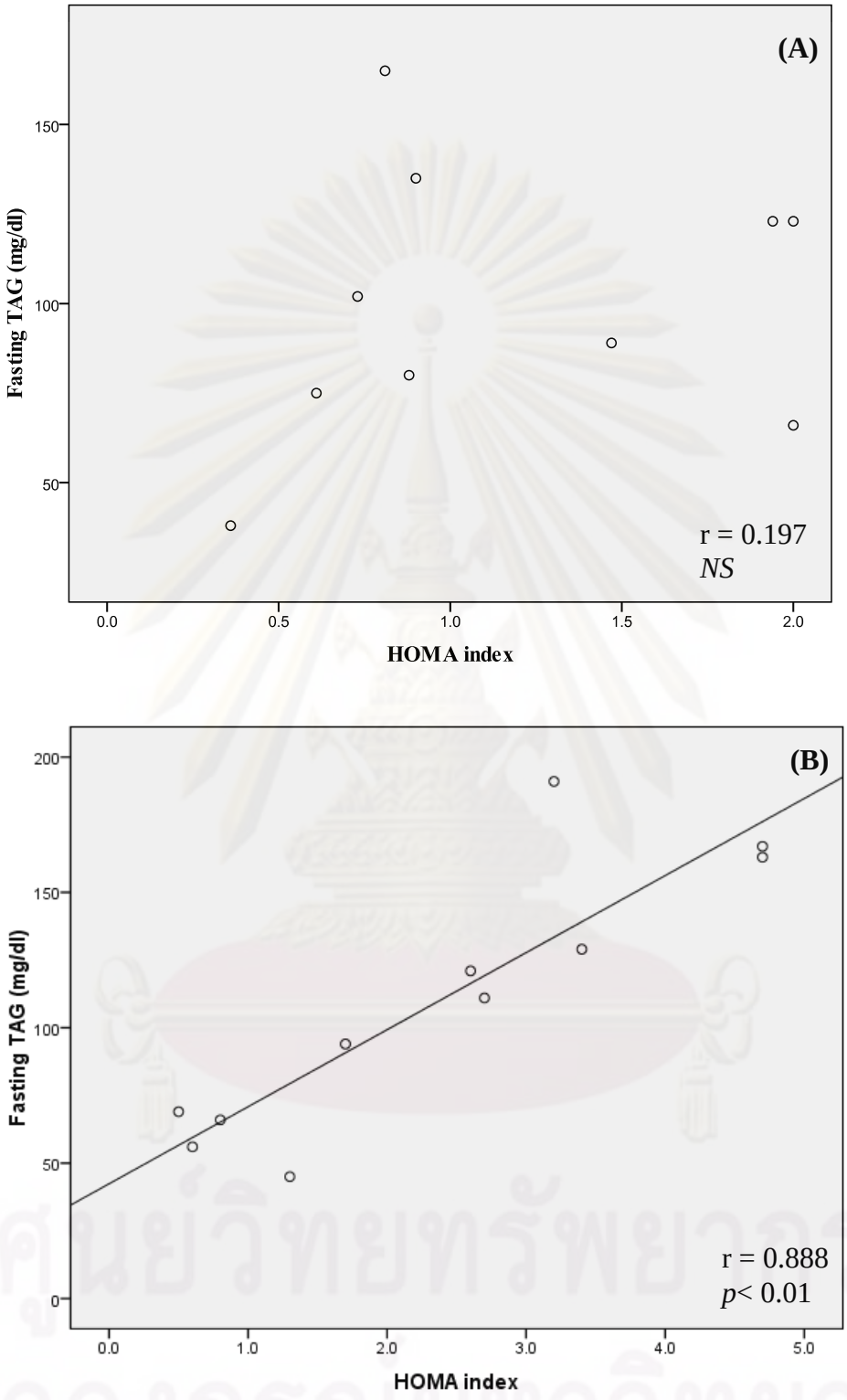


Figure 4.2 Relationship between HOMA-IR and fasting triacylglycerol level in (A) control subjects and (B) type2 diabetes subjects.

4.1.4 Determination of LDL particles size

The particles size of small dense LDL (d 1.050-1.063 g/ml) were estimated in duplicate for each sample using self casting 3-12%T nondenaturing polyacrylamide gradient gel electrophoresis. The LDL particles size were estimated from the standard curve (Figure 4.5) constructed based on the migration distances (Rf) of three markers with known diameters (first lane in Figure 4.3) and LDL standard whose diameter were determined from transmission electron microscope (Figure 4.4). Each sample was analyzed in duplicate for determining of LDL particle size.

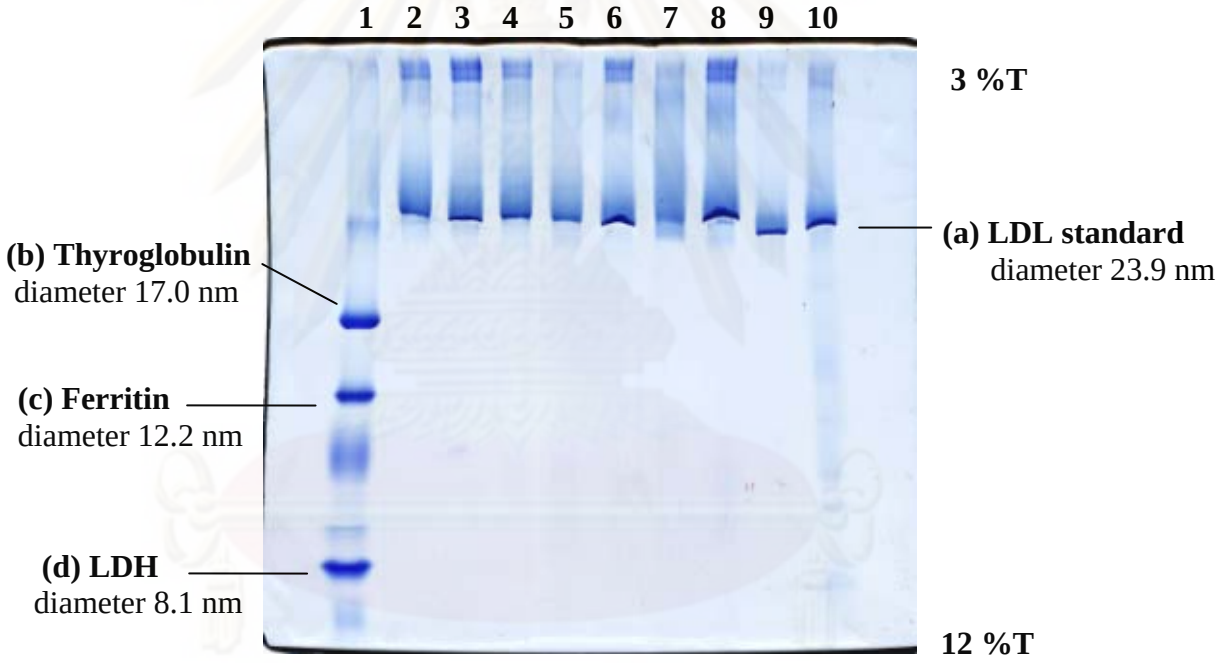


Figure 4.3 Gradient gel electrophoresis of a mixture of three markers (lane 1), LDL standard (lane 10) and LDL from subjects (lane 2-9).

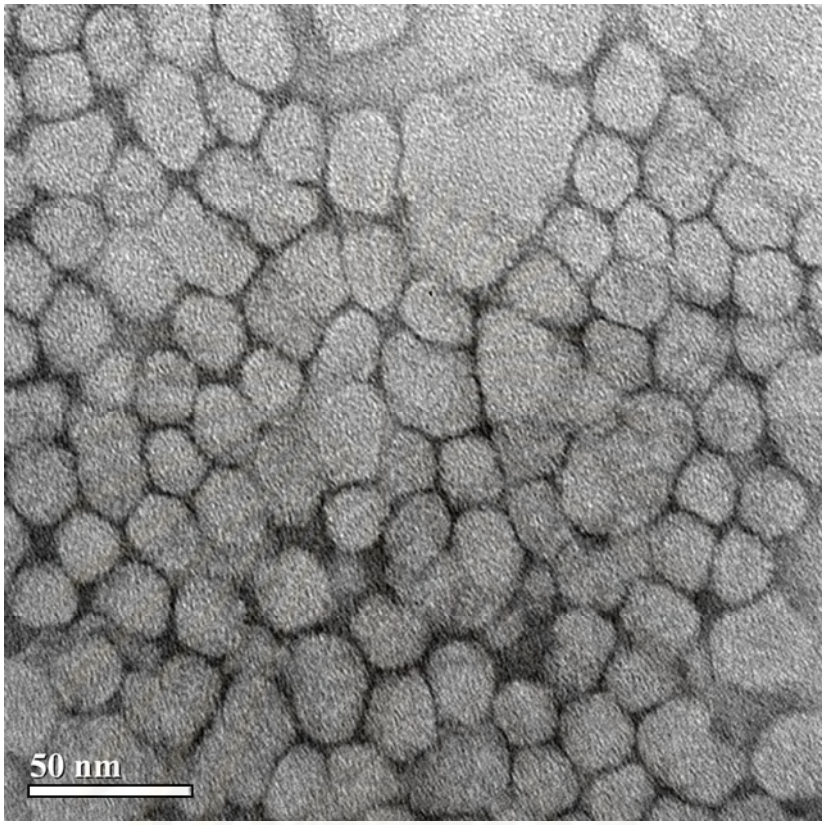


Figure 4.4 The transmission electron micrograph of LDL standard (d 1.050-1.063 g/ml). Mean of particles size was calculated from 80 particles. The mean of the LDL particles was 23.9 nm.

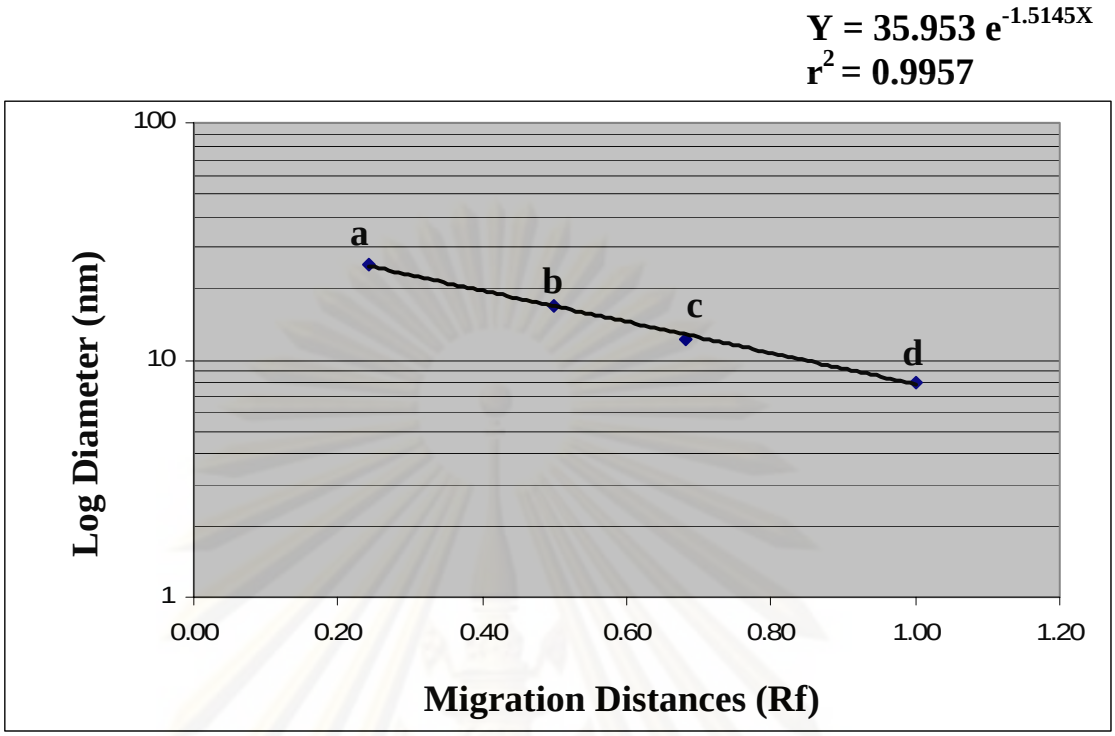


Figure 4.5 The standard curve of log diameter of four size markers versus Rf, (a) LDL standard with diameter 23.9 nm, (b) thyroglobulin with diameter 17.0 nm, (c) ferritin with diameter 12.2 nm, and (d) LDH with diameter 8.1 nm.

The small dense LDL particles size of control, LIR and HIR groups from fasting and 4 hours postprandial state were calculated and compared as shown in table 4.4.

Table 4.4 Small dense LDL particles size in fasting and 4 hours postprandial state.

Particles size (nm)	Fasting	4 hour postprandial
Control (n =10)	24.8±0.2 nm *	24.1±0.1 nm*
LIR (n=5)	24.8±0.4 nm *	23.9±0.3 nm*
HIR (n=6)	24.0±0.2 nm*†	23.5±0.3 nm*†

- Values are means ± SEM.
- * $P<0.05$ Compare the particles size between postprandial and fasting state.
- † $P<0.05$ Compare the particles size with other groups.

The particles size of small dense LDL from postprandial state was decreased in all three groups. The small dense LDL particles size of HIR diabetic group was smallest and significantly difference from control and LIR groups both fasting and postprandial state with $p<0.05$, while that of LIR group was not significantly difference from control.

It was known that hypertriacyglerolemia was considered to be an independent CVD risk factor through the small dense LDL formation and HDL reduction. Thus, the correlation between small dense LDL particles size and triacylglycerol was studied. The result of the significant correlation between postprandial triacylglycerol and postprandial small dense LDL particles size was shown in figure 4.6.

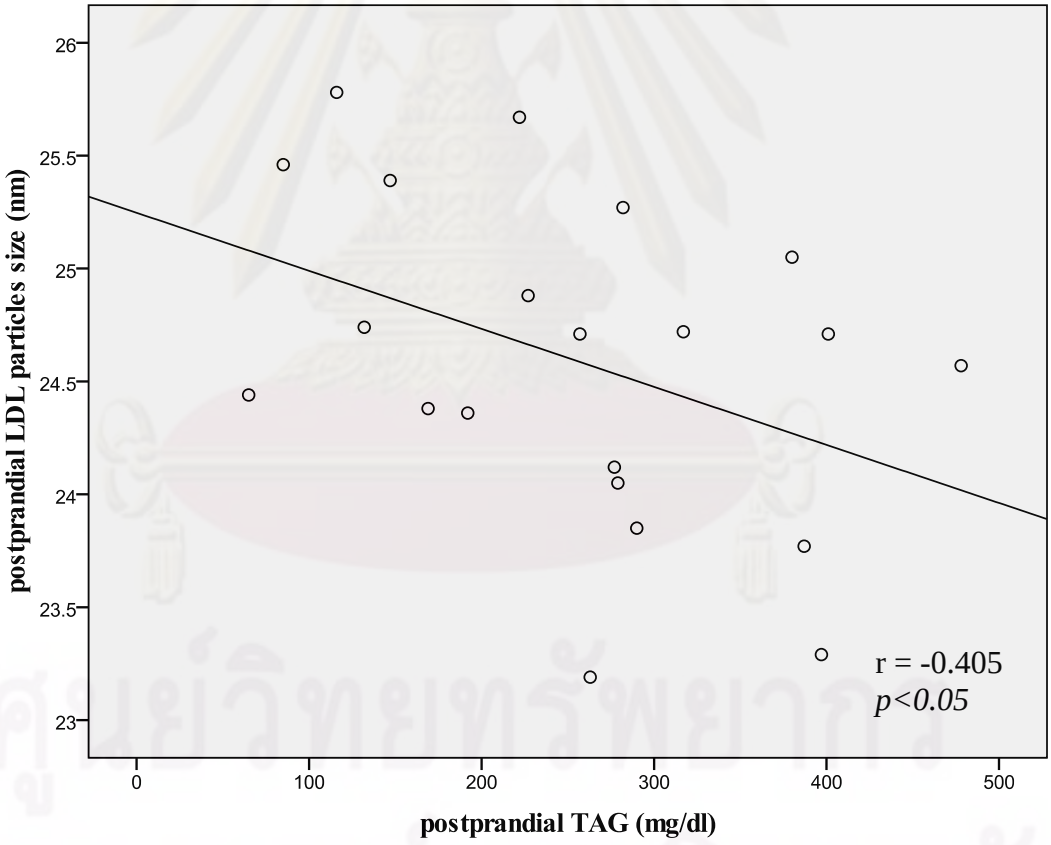


Figure 4.6 Relationship between postprandial triacylglycerol and postprandial small dense LDL particles size in all subjects.

4.1.5 Fasting and Four Hours Postprandial State Lipoprotein Profiling of Control and Diabetic Subjects

Lipoprotein profile of lipoprotein fraction; chylomicron, VLDL, LDL and HDL from fasting and postprandial of three groups of subjects were analyzed; the results were shown in table 4.5 to table 4.9.

Table 4.5 Fasting lipoproteins profile of diabetic and non diabetic subjects.

Parameters	Control (n=10)	Type 2 Diabetes (n=11)	
		HOMA-IR <2.5 (n=5)	HOMA-IR 2.5-5.0 (n=6)
Total cholesterol, mg/dl	203.5±13.6	192.2±14.3	185.8±6.3
Triacylglycerol, mg/dl	99.6±11.9	66.0±8.2	147.0±12.8
Chylomicrons			
-Cholesterol, mg/dl	0.4±0.1	0.5±0.2	0.9±0.2†*
-Triacylglycerol, mg/dl	0.8±0.3	1.8±0.4 †	2.9±0.3†*
VLDL			
-Cholesterol, mg/dl	13.6±7.0	11.6±4.2	32.5±8.6†*
-Triacylglycerol, mg/dl	66.3±13.5	34.8±17.5	127.0±12.6†*
LDL			
-Cholesterol, mg/dl	123.8±16.2	134.3±19.8	115.9±8.1†*
-Triacyglycerol, mg/dl	19.1±2.5	16.6±3.3	16.3±2.8
HDL			
-Cholesterol, mg/dl	50.4±3.6	47.4±6.9	52.6±4.9*
-Triacyglycerol, mg/dl	15.4±1.5	13.4±1.3	12.5±1.7†

Values are means ± SEM.

† $P<0.05$ compared with control.

* $P<0.05$ *vs*. LIR

From table 4.5, fasting lipoproteins profile of control and LIR were not significant differences except in chylomicron triacylglycerol (1.8±0.4 *vs.* 0.8±0.3 mg/dl, $p<0.05$) while HIR were significant differences from control in all lipoproteins fraction with $p<0.05$, except triacylglycerol level in LDL. The significant differences

(P<0.05) between LIR and HIR were observed in all lipoprotein fractions except LDL triacylglycerol and HDL triacylglycerol.

Table 4.6 Four hour postprandial lipoproteins profile of diabetic and non diabetic subjects.

Parameters	Control (n=10)	Type 2 Diabetes (n=11)	
		HOMA-IR <2.5 (n=5)	HOMA-IR 2.5-5.0 (n=6)
Total cholesterol, mg/dl	204.1±14.7	198.8±14.5 †	198.3±10.8 †
Triacylglycerol, mg/dl	239.2±32.6	164.6±34.2	358.0±31.3 †*
Chylomicrons			
-Cholesterol, mg/dl	2.6±0.5	1.8±0.7	4.5±0.8†*
-Triacylglycerol, mg/dl	88.6±20.9	58.3±14.9	159.0±30.1†*
VLDL			
-Cholesterol, mg/dl	25.4±2.9	17.4±1.9	40.8±4.2†*
-Triacylglycerol, mg/dl	103.2±6.6	70.4±7.1	171.8±8.3†*
LDL			
-Cholesterol, mg/dl	123.2±14.7	131.2±16.2	111.4±11.1†*
-Triacyglycerol, mg/dl	19.4±2.1	16.8±3.0	15.2±3.1
HDL			
-Cholesterol, mg/dl	50.9±3.6	45.3±6.6	40.6±5.2†*
-Triacyglycerol, mg/dl	21.8±1.7	15.1±2.2	20.2±3.2

Values are means ± SEM.

† P<0.05 compared with control.

* P<0.05 *vs*. LIR

From table 4.6, postprandial lipoproteins profile of control and LIR were not significantly differed in any fraction, while HIR were significantly differed (P<0.05) from control in all lipoproteins fraction except triacylglycerol level in LDL and HDL. The significant differences between LIR and HIR were observed in all lipoprotein fractions except in triacylglycerol LDL and triacylglycerol HDL.

Table 4.7 Comparative of lipoprotein profile from fasting and 4 hours postprandial in control group.

Parameters	Fasting	Postprandial
Total cholesterol, mg/dl	203.5±13.6	204.1±14.7
Triacylglycerol, mg/dl	99.6±11.9	239.2±32.6 *
Chylomicrons		
-cholesterol, mg/dl	0.4±0.1	2.6±0.5 *
-triacylglycerol, mg/dl	0.8±0.3	88.6±20.9 *
VLDL		
-cholesterol, mg/dl	13.6±7.0	25.4±2.9 *
-triacylglycerol, mg/dl	66.3±13.5	103.2±6.6 *
LDL		
-cholesterol, mg/dl	123.8±16.2	123.2±14.7
-triacylglycerol, mg/dl	19.1±2.5	19.4±2.1
HDL		
-cholesterol, mg/dl	50.4±3.6	50.9±3.6
-triacylglycerol, mg/dl	15.4±1.5	21.8±1.7 *

Values are means ± SEM.

* $P<0.05$ compared with fasting state.

From table 4.7, the significant differences with $p<0.05$ between fasting and postprandial in control subjects were observed in plasma TAG (99.6±11.9 *vs.* 239.2±32.6 mg/dl); chylomicrons cholesterol (0.4±0.1 *vs*.2.6±0.5 mg/dl) and TAG (0.8±0.3 *vs*. 88.6±20.9 mg/dl); VLDL cholesterol (13.6±7.0 *vs*.25.4±2.9 mg/dl) and TAG (66.3±13.5 vs. 103.2±6.6 mg/dl); HDL TAG (15.4±1.5 vs. 21.8±1.7 mg/dl).

Table 4.8 Comparative of lipoprotein profile from fasting and 4 hours postprandial in lower insulin resistance diabetes (LIR) group.

Parameters	Fasting	Postprandial
Total cholesterol, mg/dl	192.2±14.3	198.8±14.5
Triacylglycerol, mg/dl	66.0±8.2	164.6±34.2 *
Chylomicrons		
-cholesterol, mg/dl	0.5±0.2	1.8±0.7 *
-triacylglycerol, mg/dl	1.8±0.4	58.3±14.9 *
VLDL		
-cholesterol, mg/dl	11.6±4.2	40.8±4.2 *
-triacylglycerol, mg/dl	34.8±17.5	171.8±8.3 *
LDL		
-cholesterol, mg/dl	134.3±19.8	131.2±16.2
-triacylglycerol, mg/dl	16.6±3.3	16.8±3.0
HDL		
-cholesterol, mg/dl	47.4±6.9	45.3±6.6
-triacylglycerol, mg/dl	13.4±1.3	15.1±2.2

Values are means ± SEM.

* $P<0.05$ compared with fasting state.

From table 4.8, the significant differences with $p<0.05$ between fasting and postprandial in LIR diabetic subjects were observed in plasma TAG (66.0±8.2 *vs.* 164.6±34.2 mg/dl); chylomicrons cholesterol (0.5±0.2 *vs*.1.8±0.7 mg/dl) and TAG (1.8±0.4 *vs*. 58.3±14.9 mg/dl); VLDL cholesterol (11.6±4.2 *vs*.40.2±4.2 mg/dl).

Table 4.9 Comparative of lipoprotein profile from fasting and 4 hours postprandial in higher insulin resistance diabetes (HIR) group.

Parameters	Fasting	Postprandial
Total cholesterol, mg/dl	185.8±6.3	198.3±10.8
Triacylglycerol, mg/dl	147.0±12.8	358.0±31.3 *
Chylomicrons		
-cholesterol, mg/dl	0.9±0.2	4.5±0.8 *
-triacylglycerol, mg/dl	2.9±0.3	159.0±30.1 *
VLDL		
-cholesterol, mg/dl	32.5±8.6	40.8±4.2
-triacylglycerol, mg/dl	127.0±12.6	171.8±8.3 *
LDL		
-cholesterol, mg/dl	115.9±8.1	111.4±11.1
-triacylglycerol, mg/dl	16.3±2.8	15.2±3.1
HDL		
-cholesterol, mg/dl	52.6±4.9	40.6±5.2 *
-triacylglycerol, mg/dl	12.5±1.7	20.2±3.2 *

Values are means ± SEM.

* $P<0.05$ compared with fasting state.

From table 4.9, the significant differences with $p<0.05$ between fasting and postprandial in HIR diabetic subjects were observed in plasma TAG (147.0±12.8 *vs.* 358.0±31.3 mg/dl); chylomicrons cholesterol (0.9±0.2 *vs*. 4.5±0.8 mg/dl) and TAG (12.9±0.3 *vs*. 159.0±30.1 mg/dl); VLDL TAG (127.0±12.6 *vs*.171.8±8.3 mg/dl); HDL cholesterol (52.6±4.9 *vs*. 40.6±5.2 mg/dl) and TAG (12.5±1.7 *vs*. 20.2±3.2 mg/dl).

4.2 Experiment II: Study on the influence of insulin resistance on the alteration of apoprotein of triacylglycerol-rich lipoproteins (TRLs) between diabetes mellitus type2 and non-diabetic subjects in postprandial state.

4.2.1 Optimum Conditions for Analysis of VLDL Associated Proteins Using Two-Dimensional Electrophoresis

Our aim was using proteomics tool, two-dimensional polyacrylamide electrophoresis (2D-PAGE) and mass spectrometry to identify associated protein of triacylglycerol-rich lipoproteins (TRLs) fraction especially VLDL. So we need to find the suitable condition for VLDL associated proteins.

After VLDL fraction was prepared from sequential ultracentrifugation, delipidated with MeOH/diethyl ether system and resuspended the obtained apoproteins with thiourea rehydration buffer. The 2D-PAGE was performed using IPGphor II^{TM} system as the first dimension and PROTEAN$^{®}$ II xi cell or SE-260 mini-vertical gel electrophoresis unit as the second dimension. The result was shown in figure 4.7.

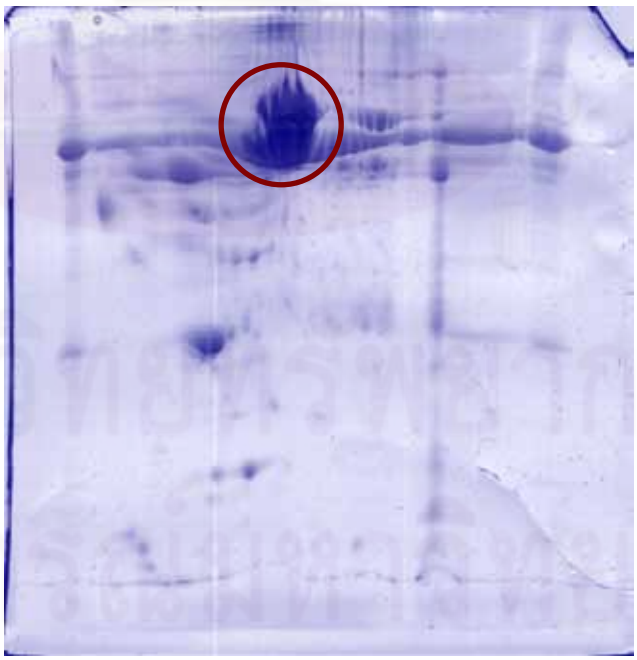


Figure 4.7 2D-PAGE pattern of TRLs associated proteins after one centrifugation step detected by colloidal coomassie blue staining.

After the one centrifuge step, the contamination of abundant protein, albumin marked in the circle was identified. To reduce the albumin contaminates recentrifugation step as the same condition was performed before lipoproteins were delipidated, and the result of two centrifugation step was shown in figure 4.8. The contamination of albumin was clearly reduced after the second centrifugation.

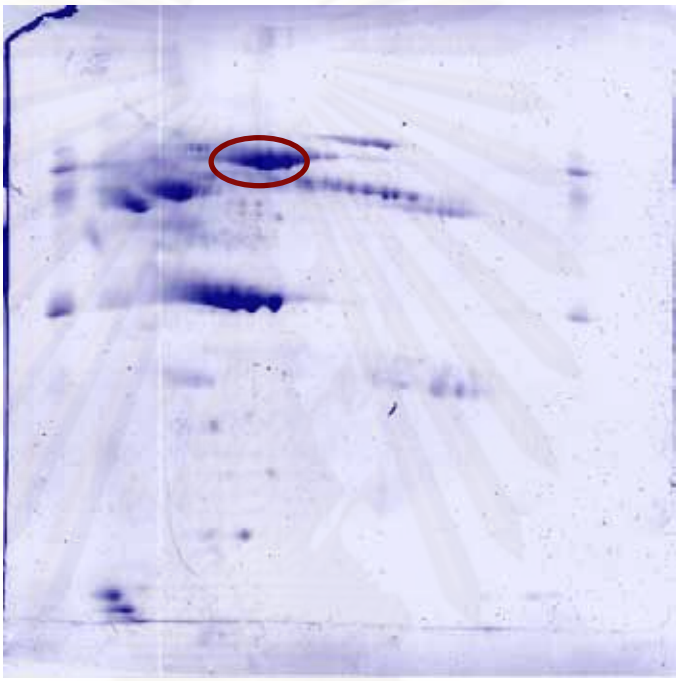


Figure 4.8 2D-PAGE pattern of TRLs associated proteins after two centrifugation steps detected by Colloidal Coomassie blue staining.

The total percentage concentration of acrylamide plus bisacrylamide in grams per 100 ml or %T affects the pore size of gel which inversely proportional to %T. %T SDS-PAGE according to Karlsson *et al*, 2005 and Mancone *et al*, 2007 was used to analyze VLDL and LDL associated proteins were 12 %T. However the results were different, Mancone and college could not separate isoform of apoC-III and apoC-II from each others, whereas Karlsson and college could. First, we decided to use the 12 %T SDS-PAGE as previous studies. The result was shown in figure 4.9.

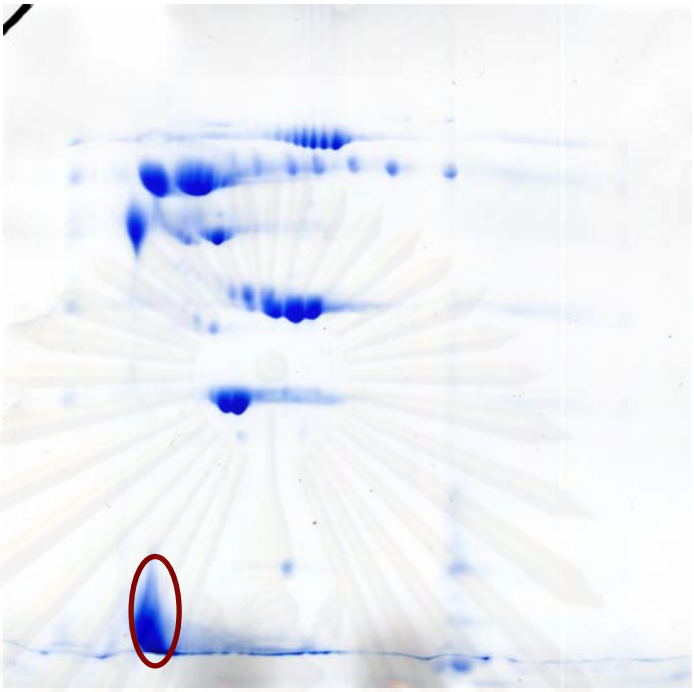


Figure 4.9 2D-PAGE pattern of TRLs associated proteins from 12%T SDS-PAGE detected by Colloidal Coomassie blue staining.

After TRLs associated proteins were separated on 12%T SDS-PAGE, stained and digested with trypsin followed with LC-MS/MS in product ion scanning by electrospray ion trap mass spectrometer, ESI-IT MS. The MS/MS results of the spot in marked circle were search against SwissProt database using Mascot search engine. The Mascot search results in figure 4.10 indicated the spot represented both apoC-III and apoC-II that mean 12%T SDS-PAGE could not separate apoC-III from apoC-II including apoC-III isoforms. So 12.5%T and 13%T SDS-PAGE were used instead 12%T SDS_PAGE and the results were shown in figure 4.11. Both 12.5%T and 13%T SDS-PAGE could separate apoC-III including isoforms as well. Finally, we decided to use 13%T SDS-PAGE in this study.

```
Email           : sangsuthum@yahoo.com
Search title    :
MS data file    : T_A1.mgf
Database        : SwissProt 57.14 (514789 sequences; 181163771 residues)
Taxonomy        : Homo sapiens (human) (20345 sequences)
Timestamp       : 23 Feb 2010 at 07:33:07 GMT
Protein hits    : APOC3_HUMAN Apolipoprotein C-III OS=Homo sapiens GN=APOC3 PE=1 SV=1
                  APOC2_HUMAN Apolipoprotein C-II OS=Homo sapiens GN=APOC2 PE=1 SV=1
                  TRY1_HUMAN  Trypsin-1 OS=Homo sapiens GN=PRSS1 PE=1 SV=1
```

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 37 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

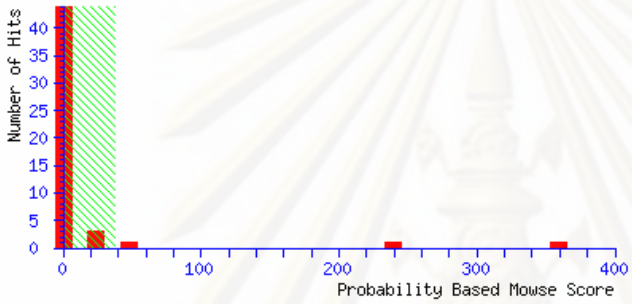


Figure 4.10 Mascot search results indicated the protein spot represented both apoC-III and apoC-II.

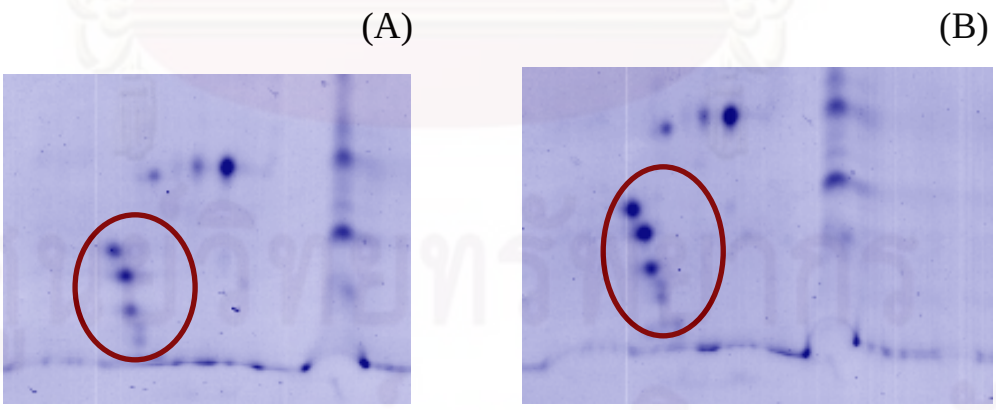


Figure 4.11 2D-PAGE pattern of TRLs associated proteins from 12.5 %T (A), and 13%T SDS-PAGE (B) the spots in gel regions represent apoC-II and apoC-III were shown in cycle.

4.2.2 VLDL Protein Profiling Using 2-DE and Mass Spectrometry

After the conditions of two-dimensional polyacrylamide electrophoresis were optimized, VLDL associated proteins were analyzed as shown in figure 4.12. Here, we run the second dimension of four strips simultaneously in PROTEAN® II xi cell (Bio-Rad, USA) to reduce the variation between run.

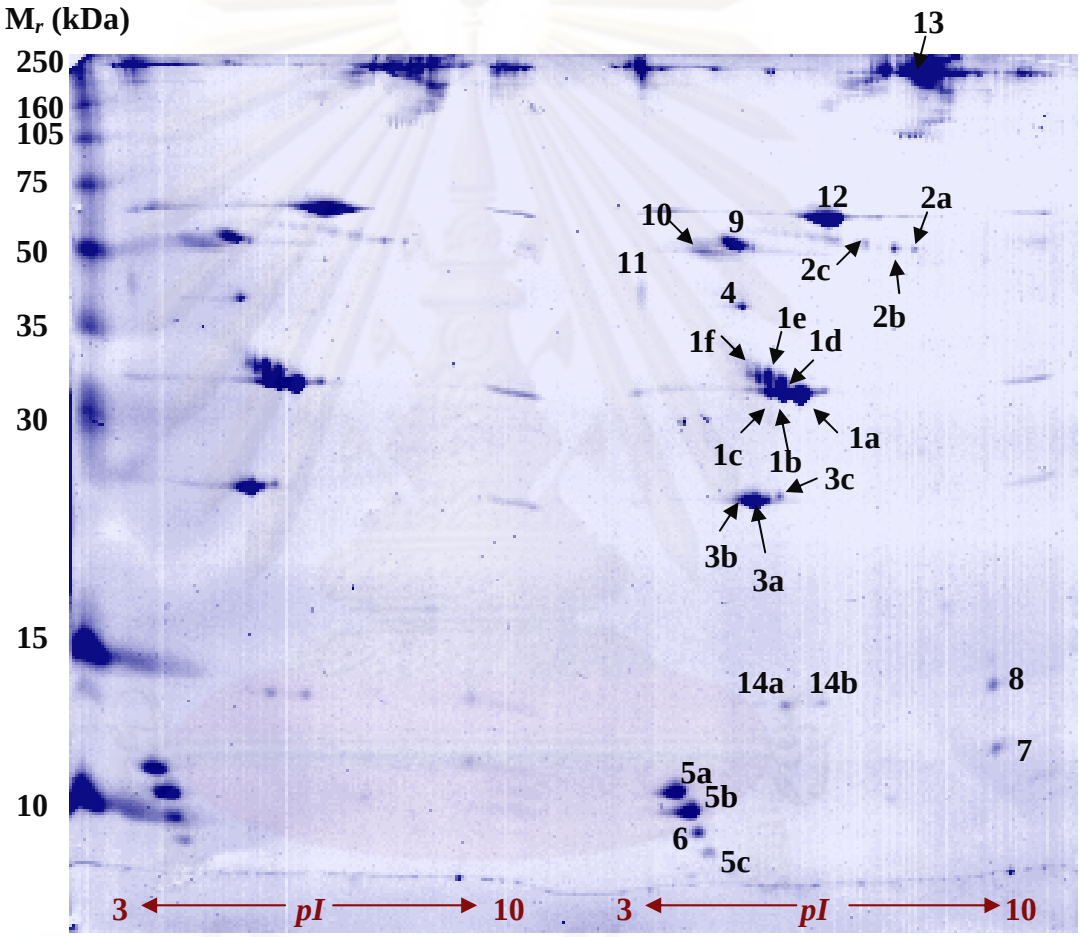


Figure 4.12 2D-PAGE patterns of VLDL associated proteins from 2 strips. 120 μg of proteins were separated with 2D-PAGE and detected by colloidal coomassie blue staining. Numbers refer to identified proteins listed in Table 4.6.

To identify VLDL associated proteins seen as spots in 2-DE, the spots were cut from gel, destained with NH_4HCO_3 and MeCN and digested with trypsin. The tryptic digest further analyzed with ESI-IT, LC-MS/MS and MALDI-TOF MS. The tandem MS analysis and peptide mass fingerprint results were search against

SwissProt database; proteins were identified as shown in table 4.10. Sequence coverage of each protein from this study was underlined which showed below and in APPENDIX D. Each letter represents 1 letter code of amino acid as shown in APPENDIX C.

Table 4.10 The identified proteins in VLDL.

Spot No.	Protein
1a-1f	apolipoprotein E
2a-2c	apolipoprotein H
3a-3c	apolipoprotein A-I
4	apolipoprotein A-IV
5a-5c	apolipoprotein C-III
6	apolipoprotein C-II
7	serum amyloid A-IV protein
8	apolipoprotein C-IV
9	α-1-antitrypsin
10	α-2-HS-glycoprotein
11	α-1-acid-glycoprotein 1
12	albumin
13	apolipoprotein B-100
14a-14b	transthyretin

ApoE sequence; matched amino acids sequence were **Underlined**

```
  1 MKVLWAALLV TFLAGCQAKV EQAVETEPEP ELRQQTEWQS GQRWELALGR
 51 FWDYLRWVQT LSEQVQEELL SSQVTQELRA LMDETMKELK AYKSELEEQL
101 TPVAEETRAR LSKELQAAQA RLGADMEDVC GRLVQYRGEV QAMLGQSTEE
151 LRVRLASHLR KLRKRLLRDA DDLQKRLAVY QAGAREGAER GLSAIRERLG
201 PLVEQGRVRA ATVGSLAGQP LQERAQAWGE RLRARMEEMG SRTRDRLDEV
251 KEQVAEVRAK LEEQAQQIRL QAEAFQARLK SWFEPLVEDM QRQWAGLVEK
301 VQAAVGTSAA PVPSDNH
```

ApoH sequence; matched amino acids sequence were **Underlined**

```
  1 MISPVLILFS SFLCHVAIAG RTCPPDDKLP FSTVVPLKTF YEPGEEITYS
 51 CKPGYVSRGG MRKFICPLTG LWPINTLKCT PRVCPFAGIL ENGAVRYTTF
101 EYPNTISFSC NTGFYLNGAD SAKCTEEGKW SPELPVCAPI ICPPPSIPTF
151 ATLRVYKPSA GNNSLYRDTA VFECLPQHAM FGNDTITCTT HGNWTKLPEC
201 REVKCPFPSR PDNGFVNYPA KPTLYYKDKA TFGCHDGYSL DGPEEIECTK
251 LGNWSAMPSC KASCKVPVKK ATVVYQGERV KIQEKFKNGM LHGDKVSFFC
301 KNKEKKCSYT EDAQCIDGTI EVPKCFKEHS SLAFWKTDAS DVKPC
```

ApoA-I sequence; matched amino acids sequence were **Underlined**

```
  1 MKAAVLTLAV LFLTGSQARH FWQQDEPPQS PWDRVKDLAT VYVDVLKDSG
 51 RDYVSQFEGS ALGKQLNLKL LDNWDSVTST FSKLREQLGP VTQEFWDNLE
101 KETEGLRQEM SKDLEEVKAK VQPYLDDFQK KWQEEMELYR QKVEPLRAEL
151 QEGARQKLHE LQEKLSPLGE EMRDRARAHV DALRTHLAPY SDELRQRLAA
201 RLEALKENGG ARLAEYHAKA TEHLSTLSEK AKPALEDLRQ GLLPVLESFK
251 VSFLSALEEY TKKLNTQ
```

ApoA-IV sequence; matched amino acids sequence were **Underlined**

```
  1 MFLKAVVLTL ALVAVAGARA EVSADQVATV MWDYFSQLSN NAKEAVEHLQ
 51 KSELTQQLNA LFQDKLGEVN TYAGDLQKKL VPFATELHER LAKDSEKLKE
101 EIGKELEELR ARLLPHANEV SQKIGDNLRE LQQRLEPYAD QLRTQVNTQA
151 EQLRRQLTPY AQRMERVLRE NADSLQASLR PHADELKAKI DQNVEELKGR
201 LTPYADEFKV KIDQTVEELR RSLAPYAQDT QEKLNHQLEG LTFQMKKNAE
251 ELKARISASA EELRQRLAPL AEDVRGNLRG NTEGLQKSLA ELGGHLDQQV
301 EEFRRRVEPY GENFNKALVQ QMEQLRQKLG PHAGDVEGHL SFLEKDLRDK
351 VNSFFSTFKE KESQDKTLSL PELEQQQEQQ QEQQQEQVQM LAPLES
```

ApoC-III sequence; matched amino acids sequence were **Underlined**

```
  1 MQPRVLLVVA LLALLASARA SEAEDASLLS FMQGYMKHAT KTAKDALSSV
 51 QESQVAQQAR GWVTDGFSSL KDYWSTVKDK FSEFWDLDPE VRPTSAVAA
```

ApoC-II sequence; matched amino acids sequence were **Underlined**

```
  1 MGTRLLPALF LVLLVLGFEV QGTQQPQQDE MPSPTFLTQV KESLSSYWES
 51 AKTAAQNLYE KTYLPAVDEK LRDLYSKSTA AMSTYTGIFT DQVLSVLKGE
101 E
```

Serum amyloid A-IV protein sequence; matched amino acids sequence were **Underlined**

```
  1 MRLFTGIVFC SLVMGVTSES WRSFFKEALQ GVGDMGRAYW DIMISNHQNS
 51 NRYLYARGNY DAAQRGPGGV WAAKLISRSR VYLQGLIDYY LFGNSSTVLE
101 DSKSNEKAEE WGRSGKDPDR FRPDGLPKKY
```

ApoC-IV sequence; matched amino acids sequence were **Underlined**

```
  1 MSLLRNRLQA LPALCLCVLV LACIGACQPE AQEGTLSPPP KLKMSRWSLV
 51 RGRMKELLET VVNRTRDGWQ WFWSPSTFRG FMQTYYDDHL RDLGPLTKAW
101 FLESKDSLLK KTHSLCPRLV CGDKDQG
```

Alpha-1-antitypsin sequence; matched amino acids sequence were **Underlined**

```
  1 MPSSVSWGIL LLAGLCCLVP VSLAEDPQGD AAQKTDTSHH DQDHPTFNKI
 51 TPNLAEFAFS LYRQLAHQSN STNIFFSPVS IATAFAMLSL GTKADTHDEI
101 LEGLNFNLTE IPEAQIHEGF QELLRTLNQP DSQLQLTTGN GLFLSEGLKL
151 VDKFLEDVKK LYHSEAFTVN FGDTEEAKKQ INDYVEKGTQ GKIVDLVKEL
201 DRDTVFALVN YIFFKGKWER PFEVKDTEEE DFHVDQVTTV KVPMMKRLGM
251 FNIQHCKKLS SWVLLMKYLG NATAIFFLPD EGKLQHLENE LTHDIITKFL
301 ENEDRRSASL HLPKLSITGT YDLKSVLGQL GITKVFSNGA DLSGVTEEAP
351 LKLSKAVHKA VLTIDEKGTE AAGAMFLEAI PMSIPPEVKF NKPFVFLMIE
401 QNTKSPLFMG KVVNPTQK
```

Alpha-2-HS-glycoprotein sequence; matched amino acids sequence were **Underlined**

```
  1 MKSLVLLLCL AQLWGCHSAP HGPGLIYRQP NCDDPETEEA ALVAIDYINQ
 51 NLPWGYKHTL NQIDEVKVWP QQPSGELFEI EIDTLETTCH VLDPTPVARC
101 SVRQLKEHAV EGDCDFQLLK LDGKFSVVYA KCDSSPDSAE DVRKVCQDCP
151 LLAPLNDTRV VHAAKAALAA FNAQNNGSNF QLEEISRAQL VPLPPSTYVE
201 FTVSGTDCVA KEATEAAKCN LLAEKQYGFC KATLSEKLGG AEVAVTCTVF
251 QTQPVTSQPQ PEGANEAVPT PVVDPDAPPS PPLGAPGLPP AGSPPDSHVL
301 LAAPPGHQLH RAHYDLRHTF MGVVSLGSPS GEVSHPRKTR TVVQPSVGAA
351 AGPVVPPCPG RIRHFKV
```

Alpha-1-acid-glycoprotein 1 sequence; matched amino acids sequence were **Underlined**

```
  1 MALSWVLTVL SLLPLLEAQI PLCANLVPVP ITNATLDQIT GKWFYIASAF
 51 RNEEYNKSVQ EIQATFFYFT PNKTEDTIFL REYQTRQDQC IYNTTYLNVQ
101 RENGTISRYV GGQEHFAHLL ILRDTKTYML AFDVNDEKNW GLSVYADKPE
151 TTKEQLGEFY EALDCLRIPK SDVVYTDWKK DKCEPLEKQH EKERKQEEGE
201 S
```

Albumin sequence; matched amino acids sequence were **Underlined**

```
  1 MKWVTFISLL FLFSSAYSRG VFRRDAHKSE VAHRFKDLGE ENFKALVLIA
 51 FAQYLQQCPF EDHVKLVNEV TEFAKTCVAD ESAENCDKSL HTLFGDKLCT
101 VATLRETYGE MADCCAKQEP ERNECFLQHK DDNPNLPRLV RPEVDVMCTA
151 FHDNEETFLK KYLYEIARRH PYFYAPELLF FAKRYKAAFT ECCQAADKAA
201 CLLPKLDELR DEGKASSAKQ RLKCASLQKF GERAFKAWAV ARLSQRFPKA
251 EFAEVSKLVT DLTKVHTECC HGDLLECADD RADLAKYICE NQDSISSKLK
301 ECCEKPLLEK SHCIAEVEND EMPADLPSLA ADFVESKDVC KNYAEAKDVF
351 LGMFLYEYAR RHPDYSVVLL LRLAKTYETT LEKCCAAADP HECYAKVFDE
401 FKPLVEEPQN LIKQNCELFE QLGEYKFQNA LLVRYTKKVP QVSTPTLVEV
451 SRNLGKVGSK CCKHPEAKRM PCAEDYLSVV LNQLCVLHEK TPVSDRVTKC
501 CTESLVNRRP CFSALEVDET YVPKEFNAET FTFHADICTL SEKERQIKKQ
551 TALVELVKHK PKATKEQLKA VMDDFAAFVE KCCKADDKET CFAEEGKKLV
601 AASQAALGL
```

ApoB-100 sequence; matched amino acids sequence were **Underlined**

```
   1 MDPPRPALLA LLALPALLLL LLAGARAEEE MLENVSLVCP KDATRFKHLR
  51 KYTYNYEAES SSGVPGTADS RSATRINCKV ELEVPQLCSF ILKTSQCTLK
 101 EVYGFNPEGK ALLKKTKNSE EFAAAMSRYE LKLAIPEGKQ VFLYPEKDEP
 151 TYILNIKRGI ISALLVPPET EEAKQVLFLD TVYGNCSTHF TVKTRKGNVA
 201 TEISTERDLG QCDRFKPIRT GISPLALIKG MTRPLSTLIS SSQSCQYTLD
 251 AKRKHVAEAI CKEQHLFLPF SYNNKYGMVA QVTQTLKLED TPKINSRFFG
 301 EGTKKMGLAF ESTKSTSPPK QAEAVLKTLQ ELKKLTISEQ NIQRANLFNK
 351 LVTELRGLSD EAVTSLLPQL IEVSSPITLQ ALVQCGQPQC STHILQWLKR
 401 VHANPLLIDV VTYLVALIPE PSAQQLREIF NMARDQRSRA TLYALSHAVN
 451 NYHKTNPTGT QELLDIANYL MEQIQDDCTG DEDYTYLILR VIGNMGQTME
 501 QLTPELKSSI LKCVQSTKPS LMIQKAAIQA LRKMEPKDKD QEVLLQTFLD
 551 DASPGDKRLA AYLMLMRSPS QADINKIVQI LPWEQNEQVK NFVASHIANI
```

601 LNSEELDIQD LKKLVKEALK ESQLPTVMDF RKFSRNYQLY KSVSLPSLDP
651 ASAKIEGNLI FDPNNYLPKE SMLKTTLTAF GFASADLIEI GLEGKGFEPT
701 LEALFGKQGF FPDSVNKALY WVNGQVPDGV SKVLVDHFGY TKDDKHEQDM
751 VNGIMLSVEK LIKDLKSKEV PEARAYLRIL GEELGFASLH DLQLLGKLLL
801 MGARTLQGIP QMIGEVIRKG SKNDFFLHYI FMENAFELPT GAGLQLQISS
851 SGVIAPGAKA GVKLEVANMQ AELVAKPSVS VEFVTNMGII IPDFARSGVQ
901 MNTNFFHESG LEAHVALKAG KLKFIIPSPK RPVKLLSGGN TLHLVSTTKT
951 EVIPPLIENR QSWSVCKQVF PGLNYCTSGA YSNASSTDSA SYYPLTGDTR
1001 LELELRPTGE IEQYSVSATY ELQREDRALV DTLKFVTQAE GAKQTEATMT
1051 FKYNRQSMTL SSEVQIPDFD VDLGTILRVN DESTEGKTSY RLTLDIQNKK
1101 ITEVALMGHL SCDTKEERKI KGVISIPRLQ AEARSEILAH WSPAKLLLQM
1151 DSSATAYGST VSKRVAWHYD EEKIEFEWNT GTNVDTKKMT SNFPVDLSDY
1201 PKSLHMYANR LLDHRVPETD MTFRHVGSKL IVAMSSWLQK ASGSLPYTQT
1251 LQDHLNSLKE FNLQNMGLPD FHIPENLFLK SDGRVKYTLN KNSLKIEIPL
1301 PFGGKSSRDL KMLETVRTPA LHFKSVGFHL PSREFQVPTF TIPKLYQLQV
1351 PLLGVLDLST NVYSNLYNWS ASYSGGNTST DHFSLRARYH MKADSVVDLL
1401 SYNVQGSGET TYDHKNTFTL SCDGSLRHKF LDSNIKFSHV EKLGNNPVSK
1451 GLLIFDASSS WGPQMSASVH LDSKKKQHLF VKEVKIDGQF RVSSFYAKGT
1501 YGLSCQRDPN TGRLNGESNL RFNSSYLQGT NQITGRYEDG TLSLTSTSDL
1551 QSGIIKNTAS LKYENYELTL KSDTNGKYKN FATSNKMDMT FSKQNALLRS
1601 EYQADYESLR FFSLLSGSLN SHGLELNADI LGTDKINSGA HKATLRIGQD
1651 GISTSATTNL KCSLLVLENE LNAELGLSGA SMKLTTNGRF REHNAKFSLD
1701 GKAALTELSL GSAYQAMILG VDSKNIFNFK VSQEGLKLSN DMMGSYAEMK
1751 FDHTNSLNIA GLSLDFSSKL DNIYSSDKFY KQTVNLQLQP YSLVTTLNSD
1801 LKYNALDLTN NGKLRLEPLK LHVAGNLKGA YQNNEIKHIY AISSAALSAS
1851 YKADTVAKVQ GVEFSHRLNT DIAGLASAID MSTNYNSDSL HFSNVFRSVM
1901 APFTMTIDAH TNGNGKLALW GEHTGQLYSK FLLKAEPLAF TFSHDYKGST
1951 SHHLVSRKSI SAALEHKVSA LLTPAEQTGT WKLKTQFNNN EYSQDLDAYN
2001 TKDKIGVELT GRTLADLTLL DSPIKVPLLL SEPINIIDAL EMRDAVEKPQ
2051 EFTIVAFVKY DKNQDVHSIN LPFFETLQEY FERNRQTIIV VVENVQRNLK
2101 HINIDQFVRK YRAALGKLPQ QANDYLNSFN WERQVSHAKE KLTALTKKYR
2151 ITENDIQIAL DDAKINFNEK LSQLQTYMIQ FDQYIKDSYD LHDLKIAIAN
2201 IIDEIIEKLK SLDEHYHIRV NLVKTIHDLH LFIENIDFNK SGSSTASWIQ
2251 NVDTKYQIRI QIQEKLQQLK RHIQNIDIQH LAGKLKQHIE AIDVRVLLDQ
2301 LGTTISFERI NDVLEHVKHF VINLIGDFEV AEKINAFRAK VHELIERYEV
2351 DQQIQVLMDK LVELTHQYKL KETIQKLSNV LQQVKIKDYF EKLVGFIDDA
2401 VKKLNELSFK TFIEDVNKFL DMLIKKLKSF DYHQFVDETN DKIREVTQRL
2451 NGEIQALELP QKAEALKLFL EETKATVAVY LESLQDTKIT LIINWLQEAL
2501 SSASLAHMKA KFRETLEDTR DRMYQMDIQQ ELQRYLSLVG QVYSTLVTYI
2551 SDWWTLAAKN LTDFAEQYSI QDWAKRMKAL VEQGFTVPEI KTILGTMPAF

2601 EVSLQALQKA TFQTPDFIVP LTDLRIPSVQ INFKDLKNIK IPSRFSTPEF
2651 TILNTFHIPS FTIDFVEMKV KIIRTIDQMQ NSELQWPVPD IYLRDLKVED
2701 IPLARITLPD FRLPEIAIPE FIIPTLNLND FQVPDLHIPE FQLPHISHTI
2751 EVPTFGKLYS ILKIQSPLFT LDANADIGNG TTSANEAGIA ASITAKGESK
2801 LEVLNFDFQA NAQLSNPKIN PLALKESVKF SSKYLRTEHG SEMLFFGNAI
2851 EGKSNTVASL HTEKNTLELS NGVIVKINNQ LTLDSNTKYF HKLNIPKLDF
2901 SSQADLRNEI KTLLKAGHIA WTSSGKGSWK WACPRFSDEG THESQISFTI
2951 EGPLTSFGLS NKINSKHLRV NQNLVYESGS LNFSKLEIQS QVDSQHVGHS
3001 VLTAKGMALF GEGKAEFTGR HDAHLNGKVI GTLKNSLFFS AQPFEITAST
3051 NNEGNLKVRF PLRLTGKIDF LNNYALFLSP SAQQASWQVS ARFNQYKYNQ
3101 NFSAGNNENI MEAHVGINGE ANLDFLNIPL TIPEMRLPYT IITTPPLKDF
3151 SLWEKTGLKE FLKTTKQSFD LSVKAQYKKN KHRHSITNPL AVLCEFISQS
3201 IKSFDRHFEK NRNNALDFVT KSYNETKIKF DKYKAEKSHD ELPRTFQIPG
3251 YTVPVVNVEV SPFTIEMSAF GYVFPKAVSM PSFSILGSDV RVPSYTLILP
3301 SLELPVLHVP RNLKLSLPHF KELCTISHIF IPAMGNITYD FSFKSSVITL
3351 NTNAELFNQS DIVAHLLSSS SSVIDALQYK LEGTTRLTRK RGLKLATALS
3401 LSNKFVEGSH NSTVSLTTKN MEVSVAKTTK AEIPILRMNF KQELNGNTKS
3451 KPTVSSSMEF KYDFNSSMLY STAKGAVDHK LSLESLTSYF SIESSTKGDV
3501 KGSVLSREYS GTIASEANTY LNSKSTRSSV KLQGTSKIDD IWNLEVKENF
3551 AGEATLQRIY SLWEHSTKNH LQLEGLFFTN GEHTSKATLE LSPWQMSALV
3601 QVHASQPSSF HDFPDLGQEV ALNANTKNQK IRWKNEVRIH SGSFQSQVEL
3651 SNDQEKAHLD IAGSLEGHLR FLKNIILPVY DKSLWDFLKL DVTTSIGRRQ
3701 HLRVSTAFVY TKNPNGYSFS IPVKVLADKF ITPGLKLNDL NSVLVMPTFH
3751 VPFTDLQVPS CKLDFREIQI YKKLRTSSFA LNLPTLPEVK FPEVDVLTKY
3801 SQPEDSLIPF FEITVPESQL TVSQFTLPKS VSDGIAALDL NAVANKIADF
3851 ELPTIIVPEQ TIEIPSIKFS VPAGIVIPSF QALTARFEVD SPVYNATWSA
3901 SLKNKADYVE TVLDSTCSST VQFLEYELNV LGTHKIEDGT LASKTKGTLA
3951 HRDFSAEYEE DGKFEGLQEW EGKAHLNIKS PAFTDLHLRY QKDKKGISTS
4001 AASPAVGTVG MDMDEDDDFS KWNFYYSPQS SPDKKLTIFK TELRVRESDE
4051 ETQIKVNWEE EAASGLLTSL KDNVPKATGV LYDYVNKYHW EHTGLTLREV
4101 SSKLRRNLQN NAEWVYQGAI RQIDDIDVRF QKAASGTTGT YQEWKDKAQN
4151 LYQELLTQEG QASFQGLKDN VFDGLVRVTQ KFHMKVKHLI DSLIDFLNFP
4201 RFQFPGKPGI YTREELCTMF IREVGTVLSQ VYSKVHNGSE ILFSYFQDLV
4251 ITLPFELRKH KLIDVISMYR ELLKDLSKEA QEVFKAIQSL KTTEVLRNLQ
4301 DLLQFIFQLI EDNIKQLKEM KFTYLINYIQ DEINTIFNDY IPYVFKLLKE
4351 NLCLNLHKFN EFIQNELQEA SQELQQIHQY IMALREEYFD PSIVGWTVKY
4401 YELEEKIVSL IKNLLVALKD FHSEYIVSAS NFTSQLSSQV EQFLHRNIQE
4451 YLSILTDPDG KGKEKIAELS ATAQEIIKSQ AIATKKIISD YHQQFRYKLQ
4501 DFSDQLSDYY EKFIAESKRL IDLSIQNYHT FLIYITELLK KLQSTTVMNP
4551 YMKLAPGELT IIL

Transthyretin sequence; matched amino acids sequence were **Underlined**

```
  1 MASHRLLLLC LAGLVFVSEA GPTGTGESKC PLMVKVLDAV RGSPAINVAV
 51 HVFRKAADDT WEPFASGKTS ESGELHGLTT EEEFVEGIYK VEIDTKSYWK
101 ALGISPFHEH AEVVFTANDS GPRRYTIAAL LSPYSYSTTA VVTNPKE
```

As shown in table 4.10, we could use proteomics tool; 2D-PAGE and mass spectrometry to identify VLDL associated proteins in overall 26 spots represented 14 different proteins and the isoforms between them were also identified. Using ESI-IT, LC-MS/MS and MALDI-Tof could identify proteins as apoE with six isoforms (spots 1a-1f), apoH with three isoforms (spots 2a-2c), apoA-I with three isoforms (spots 3a-3c), apoA-IV (spot 4), three isoforms of apoC-III (spots 5a-5c), apoC-II (spot 6), serum amyloid A-IV (spot 7), apoC-IV (spot 8), α-1-antitrypsin (spot 9), α-2-HS-glycoprotein (spot 10), α-1-acid-glycoprotein 1 (spot 11), albumin (spot 12), apoB-100 (spot 13) and two isoforms of transthyretin (spot 14a,14b). Mainly identified proteins were apoproteins and abundant contaminate proteins such as albumin, α-2-HS-glycoprotein, α-1-acid-glycoprotein which reduced by the second centrifugation step.

4.2.3 Mass Spectrometry Analysis of Peptide Modification and Polymorphism

In this study, we used MALDI-TOF MS and ESI-ion trap LC-MS/MS, to characterize the modification might occur from genetic polymorphism and post-translational modifications (PTMs). As shown in Table 4.7, MS/MS sequencing of spots represented apoE revealed our subjects are the apoE3 homozygous donor from the presence of the 1221.51, 1185.63 and 1103.6199 mass peaks which correspond to R/LGADMEDVC^{130}GR/L, R/LLRDADDLQK/R^{176} and K/R^{176}LAVYQAGAR/R, respectively. Because apoE2 has cysteine residues at both positions 130 and 176 while apoE4 and apoE5 have two arginine residues at those positions.

From MALDI spectra of apoE, the 2107.343 mass peak with 14 Da shift of 2093.344 mass peak was found as shown in Figure 4.13 and 4.14, which may

correspond to the methylated modification of 2093.344 mass peak. The MS/MS analysis of 2093.344 (Figure 4.15) and 2107.343 peaks (Figure 4.16) were used to confirm this modification including site of it. The MS/MS analysis of 2093.344 identified a AYKSELEEQLTPVAEETR peptide which corresponds to residues 91-108 of apoE, and of 2107.343 peak identified a modified AYKSELEE(Me)QLTPVAEETR peptide result from methylated modification on glutamic acid residue (E).

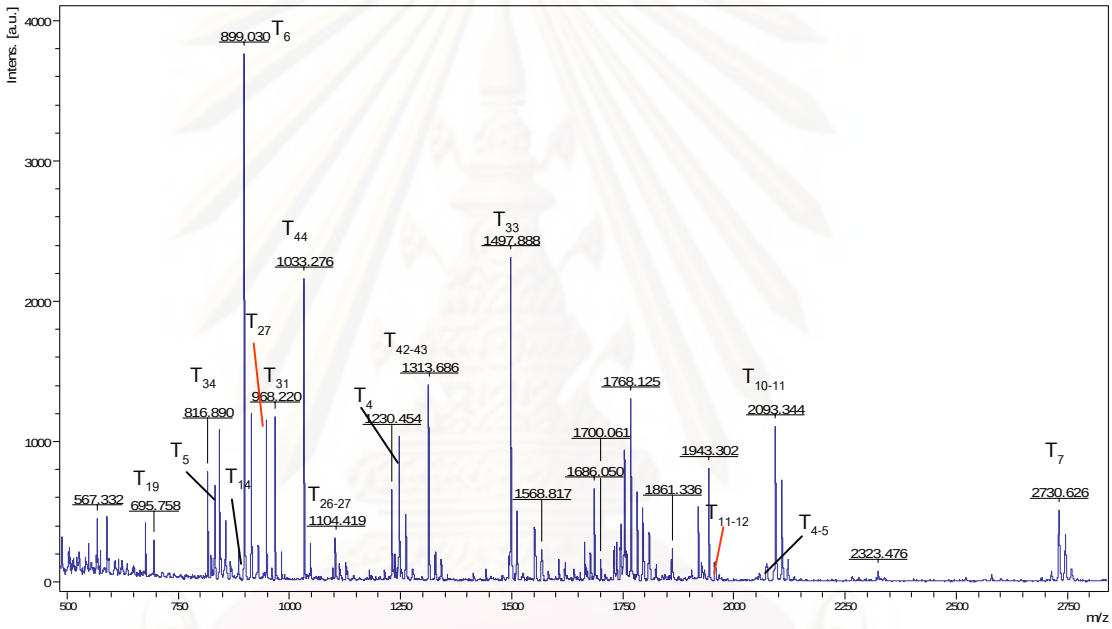


Figure 4.13 MALDI-ToF mass spectrum of tryptic peptides of apoE.

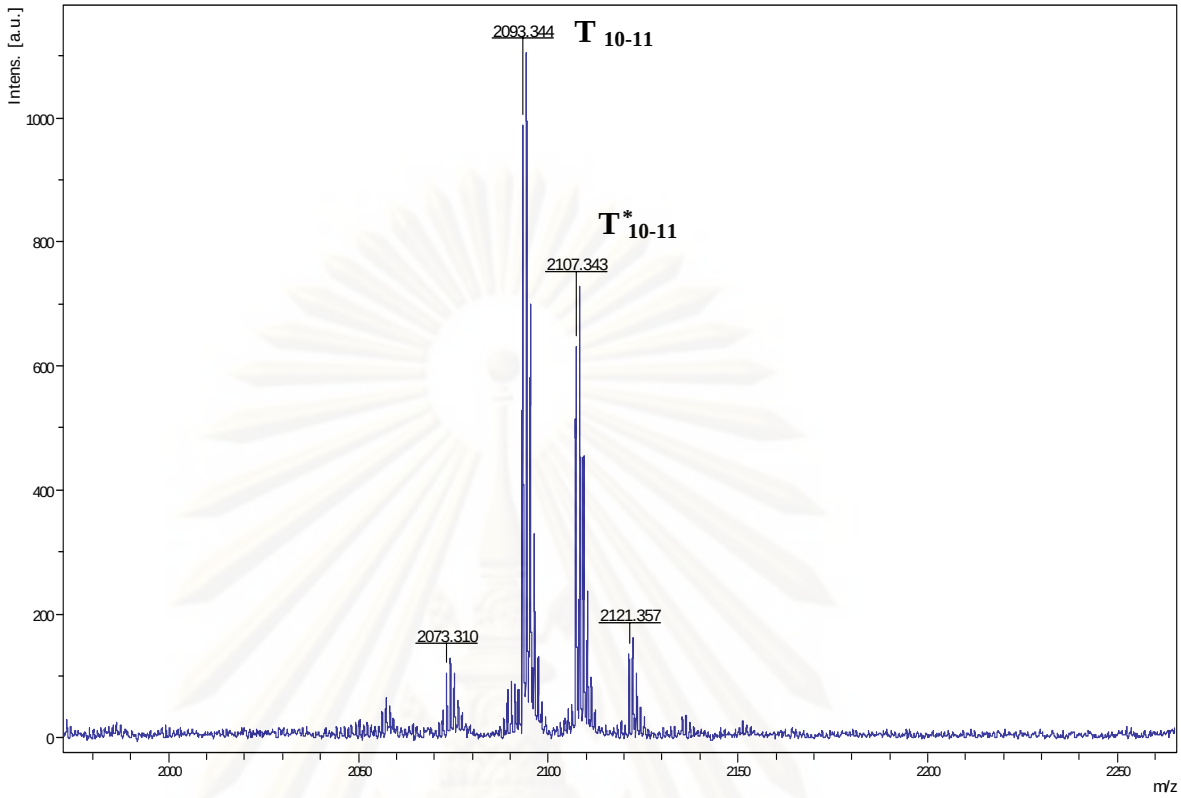


Figure 4.14 MALDI-ToF mass spectrum of tryptic peptides of apoE zoom in the peaks 2093.344 m/z and 2107.343 m/z represent $T_{10\text{-}11}$ and modified peptide, $T^{*}_{10\text{-}11}$.

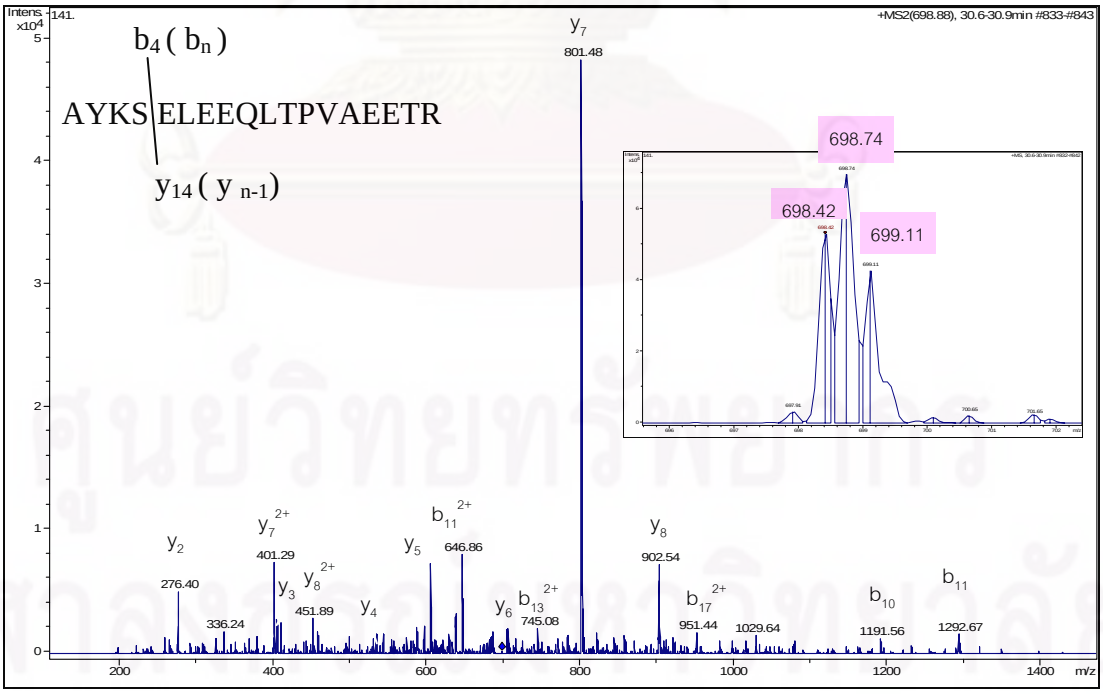


Figure 4.15 Product ion scanning mass spectrum of 698.42, $[M+3H]^{3+}$ represent $T_{10\text{-}11}$ (2093.34, $[M+H]^{+}$) of apoE.

The mass spectrum in figure 4.15 presents the mass peaks of b and y ions represent sequence of AYKSELEEQLTPVAEETR peptide as shown in table 4.11 in bold.

Table 4.11 Calculated masses of AYKSELEEQLTPVAEETR peptide.

N-Term.	Ion	b	y	C-Term.	Ion
1	A	72.04	175.12	R	1
2	Y	235.11	**276.17**	T	2
3	K	363.20	**405.21**	E	3
4	S	450.24	**534.25**	E	4
5	E	579.28	**605.29**	A	5
6	L	692.36	**704.36**	V	6
7	E	821.40	**801.41**	P	7
8	E	950.45	**902.46**	T	8
9	Q	1078.51	1015.54	L	9
10	L	**1191.59**	1143.60	Q	10
11	T	**1292.64**	1272.64	E	11
12	P	1389.69	1401.67	E	12
13	V	**1488.76**	1514.77	L	13
14	A	1559.80	1643.81	E	14
15	E	1688.84	1730.84	S	15
16	E	1817.88	1858.94	K	16
17	T	1918.93	2022.00	Y	17
18	R	2075.03	2093.04	A	18

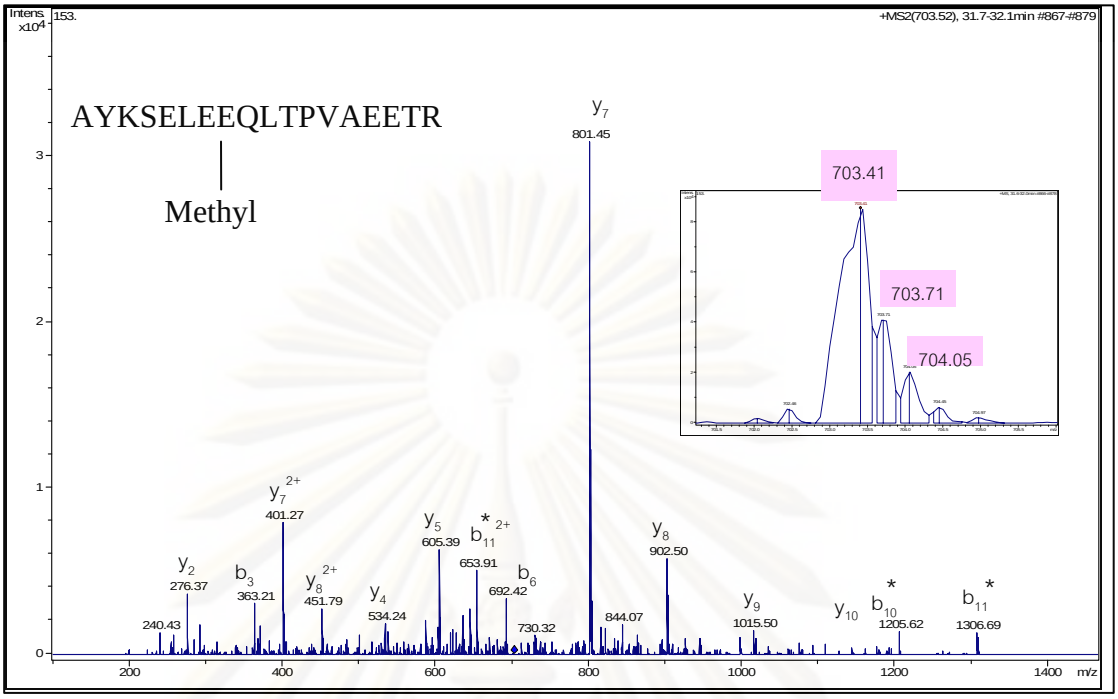


Figure 4.16 Product ion scanning mass spectrum of 703.41, $[M+3H]^{3+}$ represent methylated of $T_{10\text{-}11}$ (2107.34, $[M+H]^{+}$) of apoE. The b_n* ions represent the methylated modification.

The mass spectrum in figure 4.16 presents the mass peaks of b and y ions represent sequence of methylated AYKSELEE(Me)QLTPVAEETR peptide on glutamic acid residue at position b_8 or y_{11}. Thus, since b_8 ion to b_{18} ion and y_{11} ion to y_{18} ion mass were shift 14 Da from unmodified peptide. The mass peaks of b and y ions represent sequence of the peptide were shown in table 4.12 in bold.

Table 4.12 Calculated masses of AYKSELEE(Me)QLTPVAEETR peptide.

N-Term.	Ion	b	y	C-Term.	Ion
1	A	72.04	175.12	R	1
2	Y	235.11	**276.17**	T	2
3	K	**363.20**	**405.21**	E	3
4	S	450.24	**534.25**	E	4
5	E	579.28	**605.29**	A	5
6	L	**692.36**	704.36	V	6
7	E	821.40	**801.41**	P	7
8	E	964.45	**902.46**	T	8
9	Q	1092.51	**1015.54**	L	9
10	L	**1205.59**	**1143.60**	Q	10
11	T	**1306.64**	1286.64	E	11
12	P	1403.69	1415.67	E	12
13	V	1502.76	1528.77	L	13
14	A	1573.80	1657.81	E	14
15	E	1702.84	1744.84	S	15
16	E	1831.88	1872.94	K	16
17	T	1932.93	2036.00	Y	17
18	R	2089.03	2107.04	A	18

4.2.4 VLDL Protein Profiling of Type2 Diabetes Mellitus

To study the influence of insulin resistance on the pattern of TRLs proteins, six pools of VLDL were prepared from 12 hours fasting and 4 hours postprandial plasma of type2 diabetes with varied insulin resistance and non-diabetes subjects using sequential ultracentrifugation. After delipidation, the VLDL proteins were analyzed with 2D-PAGE in triplicate for each pool, detected with colloidal coomassie blue staining and analyzed with ImageMaster 2D Platinum^{TM} software. First, reference gel was set for compared to the others gel. After gels were compared, each spot of protein was given a number; the same number in every gel represented the same protein. The significantly difference of each spot was compare using *t-test* analysis, $p<0.05$.

The histograms of spots significantly difference between control and HIR group from 4 hours postprandial VLDL were shown in figure 4.17, letter “a” means the mean relative amount (%) of VLDL associated proteins in control group; “b” means the mean relative amount (%) of VLDL associated proteins in HIR group. The identified proteins of each numbers were shown in table 4.13.

The different amount of VLDL-associated proteins between non diabetes and both of diabetic groups from fasting and 4 hours postprandial VLDL were calculated from the mean relative amount (%) of each protein and shown in figure 4.18 to figure 4.21 and table 4.14 to table 4.16. Only proteins have the significant difference at $p<0.05$ using Student’s *t*-test analysis were labeled with asterisk (*) in figures.

The different amount of VLDL-associated proteins between fasting and 4 hours within individual groups were calculated from the mean relative amount (%) of each protein and shown in figure 4.22 to figure 4.24 and table 4.17 to 4.19. Only proteins have the significant difference at $p<0.05$ using Student’s *t*-test analysis were labeled with asterisk (*) in figures.

Every gel had the same pattern of VLDL proteins however the relative amount of some proteins were different. Interesting, transthyretin which has not been identified in VLDL and correlate to insulin resistance was found higher amount than

control in both diabetic postprandial and in HIR diabetic fasting. ApoA-IV was found lower amount only in fasting state in diabetic group especially HIR. ApoA-I major isoform was found lower amount in both fasting and postprandial state in HIR than LIR compare to control. ApoE was found more increasing in control group in postprandial state and lower amount in HIR group. ApoC-III was found more abundant in HIR and LIR than control in fasting state and more increasing in postprandial state.

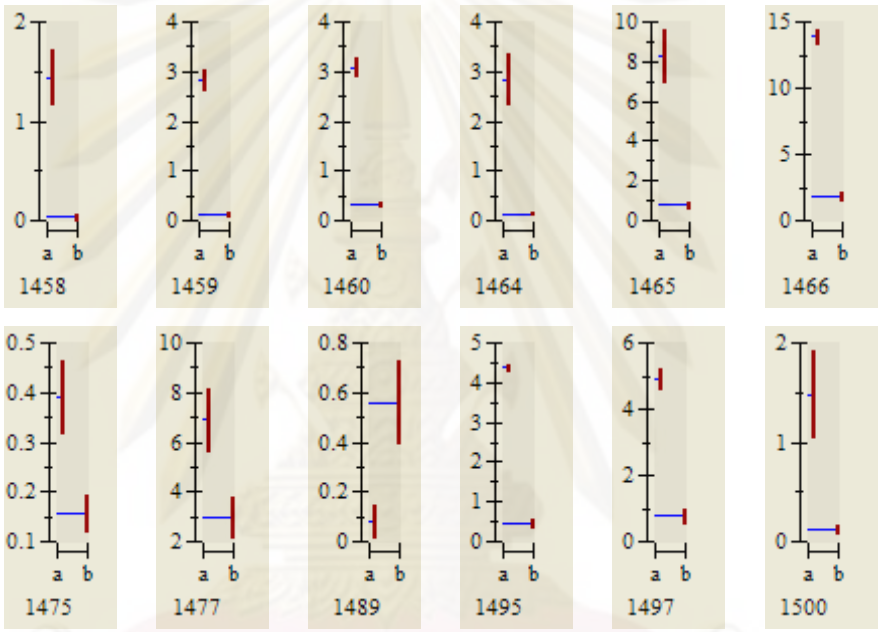


Figure 4.17 Histogram of spots significantly difference between control and HIR group from postprandial VLDL, “a” was control, “b” was HIR.

Table 4.13 The identified proteins of each numbers from figure 4.17.

Spot Number	Identified Protein
1458	glycosylated apoE acidic isoform
1459	glycosylated apoE isoform
1460	glycosylated apoE basic isoform
1464	non-glycosylated apoE acidic isoform
1465	non-glycosylated apoE isoform
1466	non-glycosylate apoE basic isoform
1475	pro apoA-I
1477	apoA-I base or apoA-I major isoform
1489	Transthyretin, TTR
1495	apoC-III_2
1497	apoC-III_1
1500	apoC-II

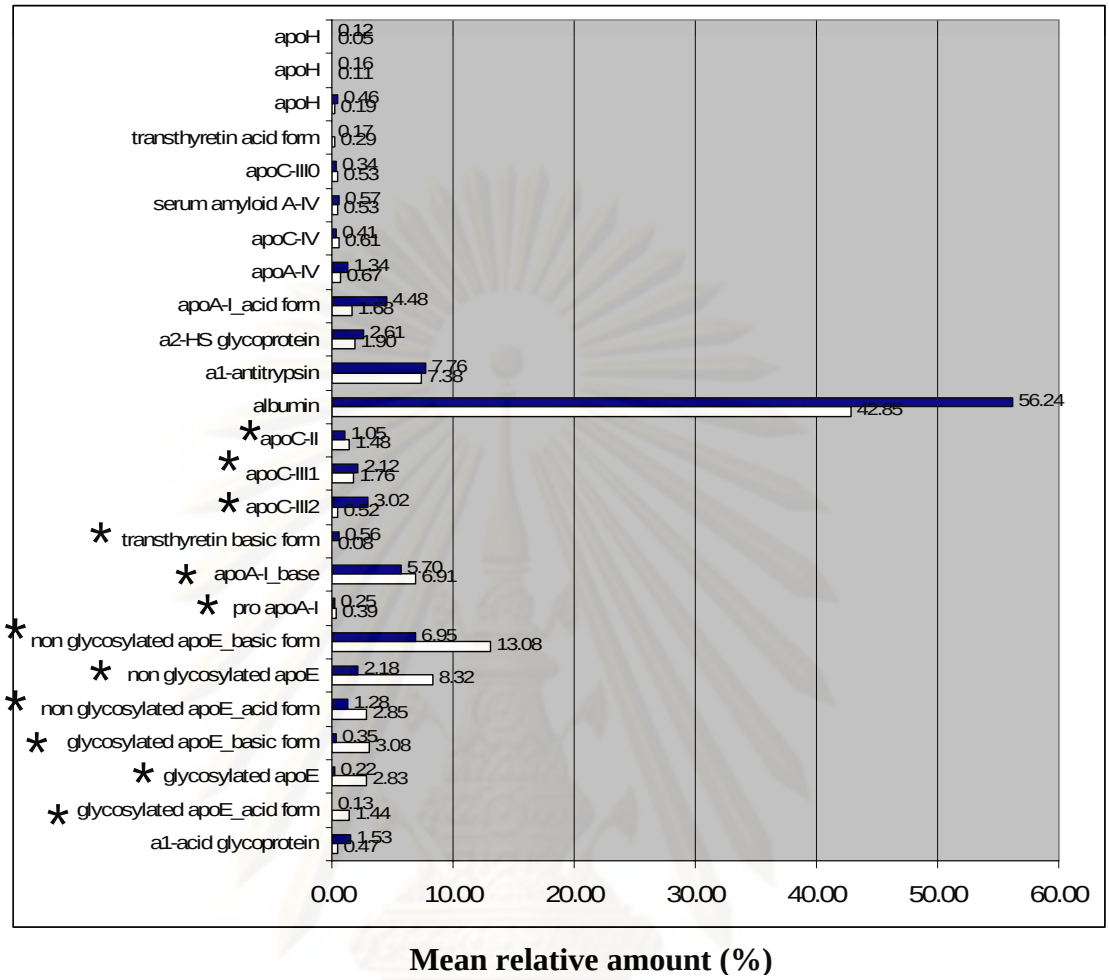


Figure 4.18 Quantitative differences in VLDL-associated proteins between control (white bar) and LIR (black bar) from 4 hours postprandial VLDL were calculated from the mean relative amount (%) of each protein. ***** $P<0.05$ compared with control.

The relative amounts of all isoforms of apoE, pro apoA-I, major isoform of apoA-I (apoA-I base) and apoC-II were significantly less in LIR subjects. On the other hand, apoC-III_1 and apoC-III_2 were significantly more in LIR subjects.

Table 4.14 The alteration of VLDL proteins from postprandial state between control and LIR group.

Proteins	Increase	Decrease
α1-acid glycoprotein	↑	
glycosylated apoE_acid form		↓
glycosylated apoE		↓
glycosylated apoE_basic formnon		↓
glycosylated apoE_acid form non		↓
glycosylated apoE		↓
non glycosylated apoE_basic form		↓
pro apoA-I		↓
apoA-I_base		↓
transthyretin basic form	↑	
apoC-III_2	↑	
apoC-III_1	↑	
apoC-II		↓
albumin	↑	
α1-antitrypsin	↑	
α2-HS glycoprotein	↑	
apoA-I_acid form	↑	
apoA-IV	↑	
apoC-IV		↓
serum amyloid A-IV	↑	
apoC-III_0		↓
transthyretin acid form		↓
apoH	↑	
apoH	↑	
apoH	↑	

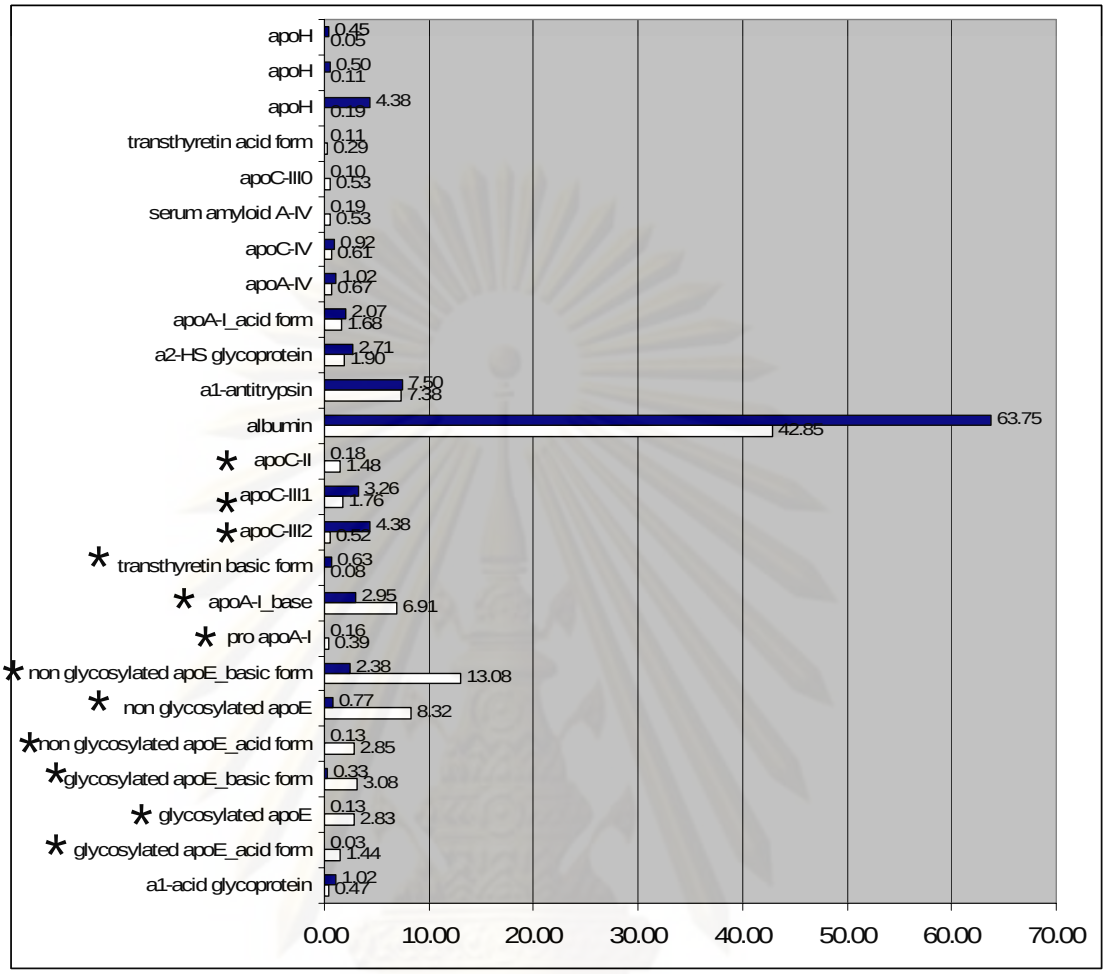


Figure 4.19 Quantitative differences in VLDL-associated proteins between control (white bar) and HIR (black bar) from 4 hours postprandial VLDL were calculated from the mean relative amount (%) of each protein. ***** $P<0.05$ compared with control.

The relative amounts of all isoforms of apoE, pro apoA-I, major isoform of apoA-I (apoA-I base) and apoC-II were significantly less in HIR subjects. On the other hand, apoC-III_1 and apoC-III_2 and transthyretin basic isoform were significantly more in HIR subjects.

Table 4.15 The alteration of VLDL proteins from postprandial state between control and HIR group.

Proteins	Increase	Decrease
α1-acid glycoprotein	↑	
glycosylated apoE_acid form		↓
glycosylated apoE		↓
glycosylated apoE_basic formnon		↓
glycosylated apoE_acid form non		↓
glycosylated apoE		↓
non glycosylated apoE_basic form		↓
pro apoA-I		↓
apoA-I_base		↓
transthyretin basic form	↑	
apoC-III_2	↑	
apoC-III_1	↑	
apoC-II		↓
albumin	↑	
α1-antitrypsin	↑	
α2-HS glycoprotein	↑	
apoA-I_acid form	↑	
apoA-IV	↑	
apoC-IV	↑	
serum amyloid A-IV		↓
apoC-III_0		↓
transthyretin acid form		↓
apoH	↑	
apoH	↑	
apoH	↑	

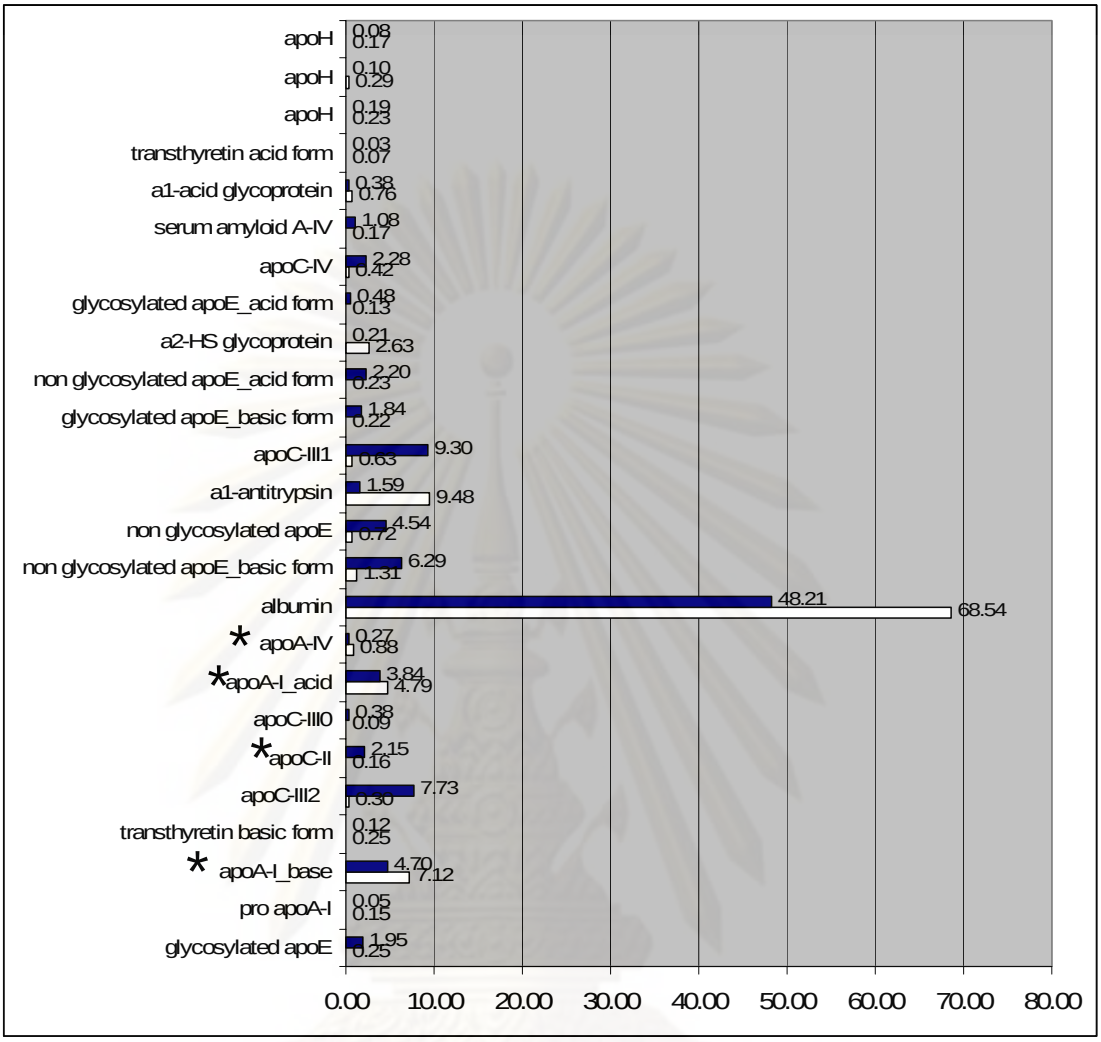


Figure 4.20 Quantitative differences in VLDL-associated proteins between control (white bar) and LIR (black bar) from fasting VLDL were calculated from the mean relative amount (%) of each protein. ***** $P<0.05$ compared with control.

The relative amounts of major isoform of apoA-I (apoA-I base), apoA-I acid isoform and apoA-IV were significantly less in LIR subjects, whereas apoC-II was significantly more in LIR subjects.

Table 4.16 The alteration of VLDL proteins from fasting state between control and LIR group.

Proteins	Increase	Decrease
glycosylated apoE	↑	
pro apoA-I		↓
apoA-I_base		↓
transthyretin basic form		↓
apoC-III_2	↑	
apoC-II	↑	
apoC-III_0	↑	
apoA-I_acid		↓
apoA-IV		↓
albumin		↓
non glycosylated apoE_basic	↑	
formnon glycosylated apoE	↑	
α1-antitrypsin		↓
apoC-III_1	↑	
glycosylated apoE_basic form	↑	
non glycosylated apoE_acid form	↑	
α2-HS glycoprotein	↑	
glycosylated apoE_acid form	↑	
apoC-IV	↑	
serum amyloid A-IV	↑	
α1-acid glycoprotein		↓
transthyretin acid form		↓
apoH		↓
apoH		↓
apoH		↓

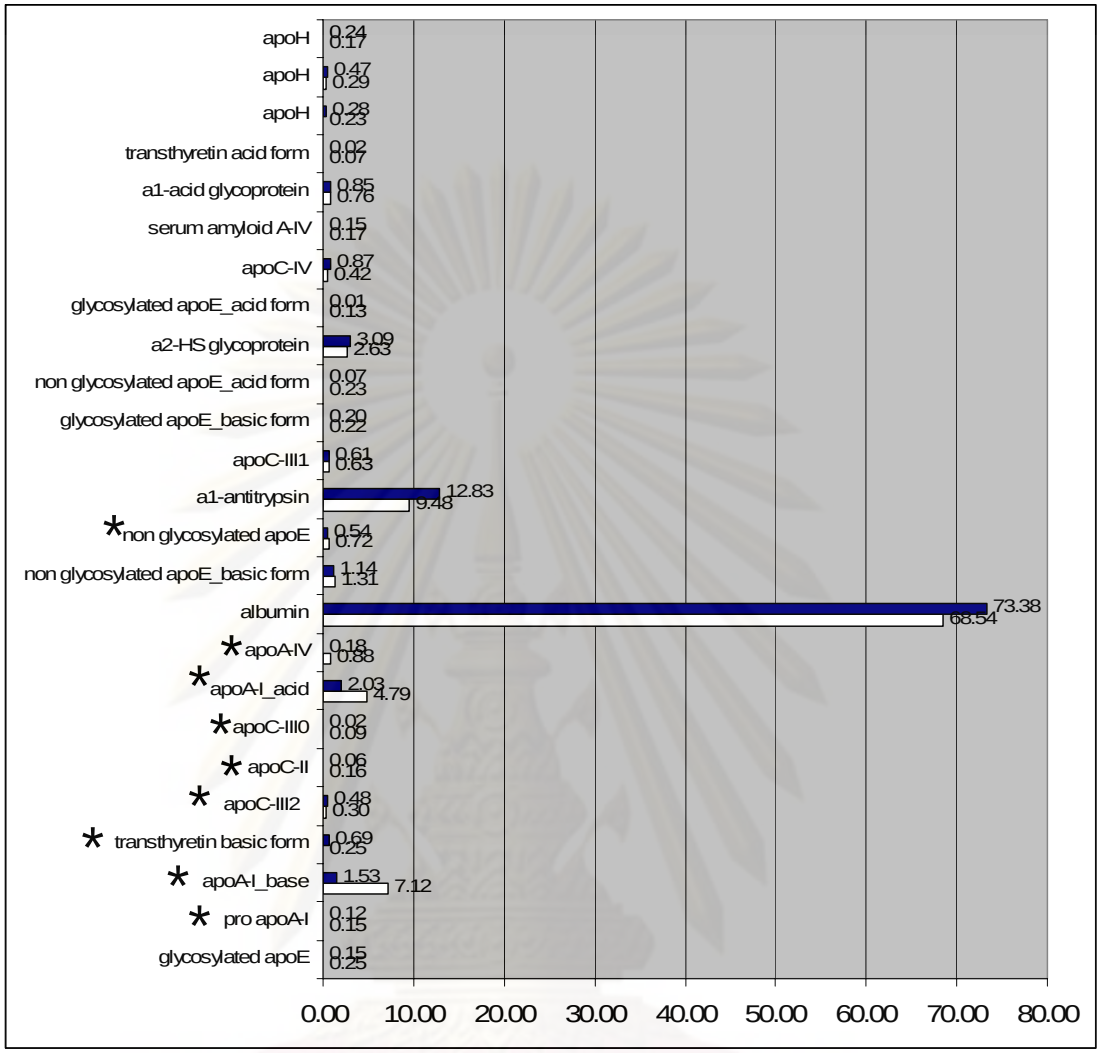


Figure 4.21 Quantitative differences in VLDL-associated proteins between control (white bar) and HIR (black bar) from fasting VLDL were calculated from the mean relative amount (%) of each protein. ***** $P<0.05$ compared with control.

The relative amounts of all isoform of apoA-I, apoA-IV, apoC-II, apoC-III_0 and non glycosylated apoE were significantly less in HIR subjects, whereas transthyretin basic isoform was significantly more in HIR subjects.

Table 4.17 The alteration of VLDL proteins from fasting state between control and HIR group.

Proteins	Increase	Decrease
glycosylated apoE		↓
pro apoA-I		↓
apoA-I_base		↓
transthyretin basic form	↑	
apoC-III_2	↑	
apoC-II		↓
apoC-III_0		↓
apoA-I_acid		↓
apoA-IV		↓
albumin	↑	
non glycosylated apoE_basic		↓
formnon glycosylated apoE		↓
α1-antitrypsin	↑	
apoC-III_1		↓
glycosylated apoE_basic form		↓
non glycosylated apoE_acid form		↓
α2-HS glycoprotein	↑	
glycosylated apoE_acid form		↓
apoC-IV		↓
serum amyloid A-IV		↓
α1-acid glycoprotein	↑	
transthyretin acid form		↓
apoH	↑	
apoH	↑	
apoH	↑	

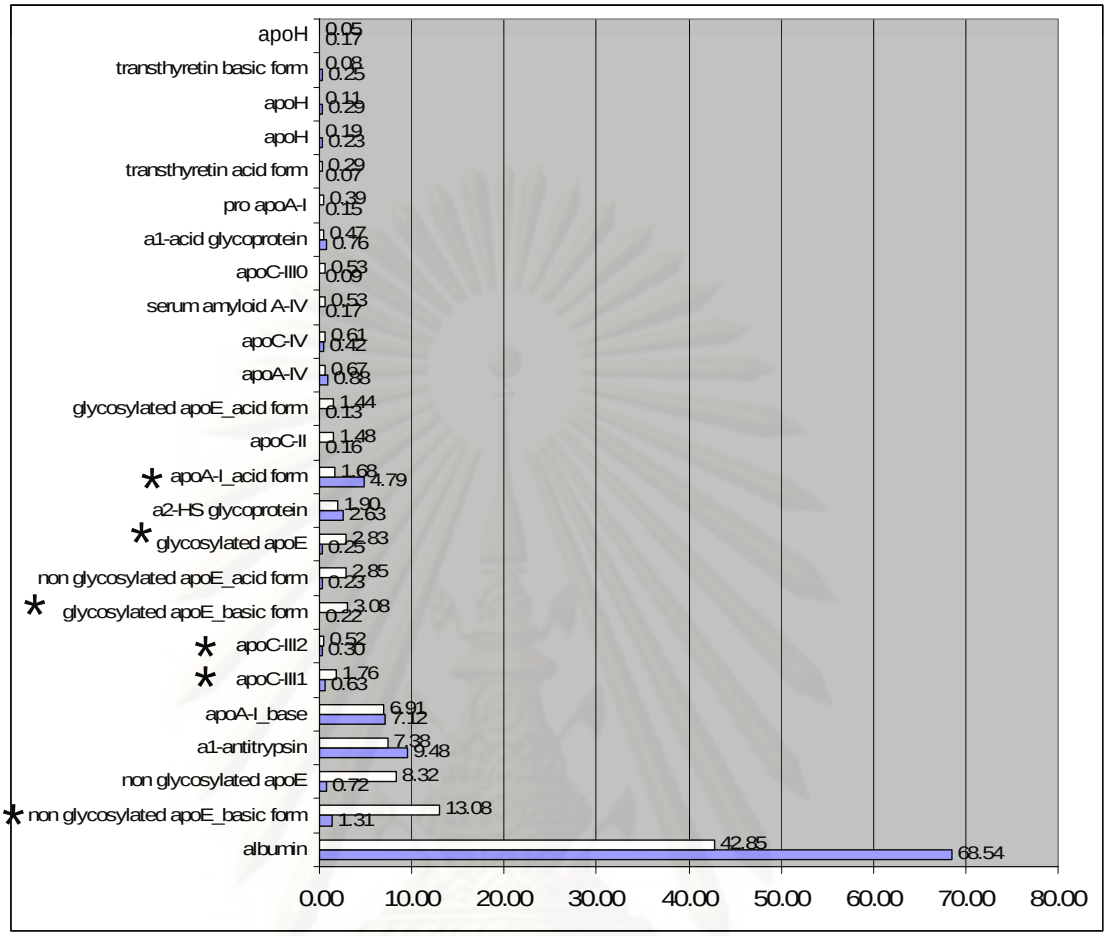


Figure 4.22 Quantitative differences in VLDL-associated proteins between postprandial state (white bar) and fasting state (black bar) from control group were calculated from the mean relative amount (%) of each protein. ***** $P<0.05$ compared with fasting state.

The relative amounts of apoA-I acid isoform and α-2-HS glycoprotein were significantly less in postprandial state, whereas glycosylated apoE, non glycosylated apoE basic isoform, glycosylated apoE basic isoform, apoC-III_1 and apoC-III_2 were significantly more in postprandial state.

Table 4.18 The alteration of VLDL proteins from fasting to postprandial state in control group.

Proteins	Increase	Decrease
albumin		↓
non glycosylated apoE_basic form	↑	
non glycosylated apoE	↑	
α1-antitrypsin		↓
apoA-I_base		↓
apoC-III_1	↑	
apoC-III_2	↑	
glycosylated apoE_basic form	↑	
non glycosylated apoE_acid form	↑	
glycosylated apoE	↑	
α2-HS glycoprotein		↓
apoA-I_acid form		↓
apoC-II	↑	
glycosylated apoE_acid form	↑	
apoA-IV		↓
apoC-IV	↑	
serum amyloid A-IV	↑	
apoC-III_0	↑	
α1-acid glycoprotein		↓
pro apoA-I	↑	
transthyretin acid form	↑	
apoH		↓
apoH		↓
transthyretin basic form		↓
apoH		↓

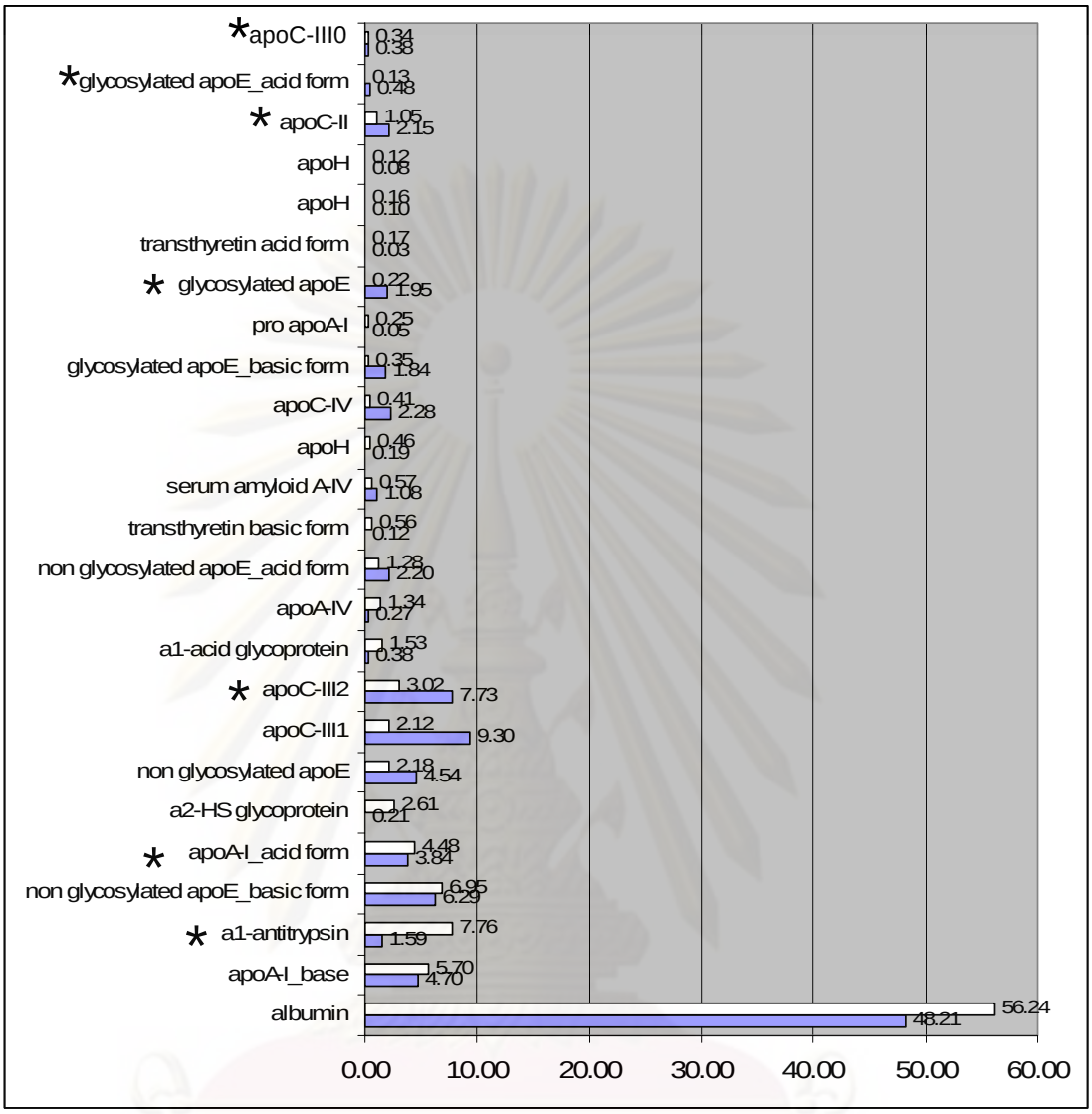


Figure 4.23 Quantitative differences in VLDL-associated proteins between postprandial state (white bar) and fasting state (black bar) from LIR group were calculated from the mean relative amount (%) of each protein. ***** $P<0.05$ compared with fasting state.

The relative amounts of apoC-III_2, apoC-III_0, apoC-II, glycosylated apoE and glycosylated apoE acid isoform were significantly less in postprandial state, whereas α-1-antitrypsin, apoA-I acid isoform were significantly more in postprandial state.

Table 4.19 The alteration of VLDL proteins from fasting to postprandial state in lower insulin resistance diabetes (LIR) group.

Proteins	Increase	Decrease
albumin	↑	
apoA-I_base	↑	
α1-antitrypsin	↑	
non glycosylated apoE_basic form	↑	
apoA-I_acid form	↑	
α2-HS glycoprotein	↑	
non glycosylated apoE		↓
apoC-III_1		↓
apoC-III_2		↓
α1-acid glycoprotein	↑	
apoA-IV	↑	
non glycosylated apoE_acid form		↓
transthyretin basic form	↑	
serum amyloid A-IV		↓
apoH	↑	
apoC-IV		↓
glycosylated apoE_basic form		↓
pro apoA-I	↑	
glycosylated apoE		↓
transthyretin acid form	↑	
apoH	↑	
apoH	↑	
apoC-II		↓
glycosylated apoE_acid form		↓
apoC-III_0		↓

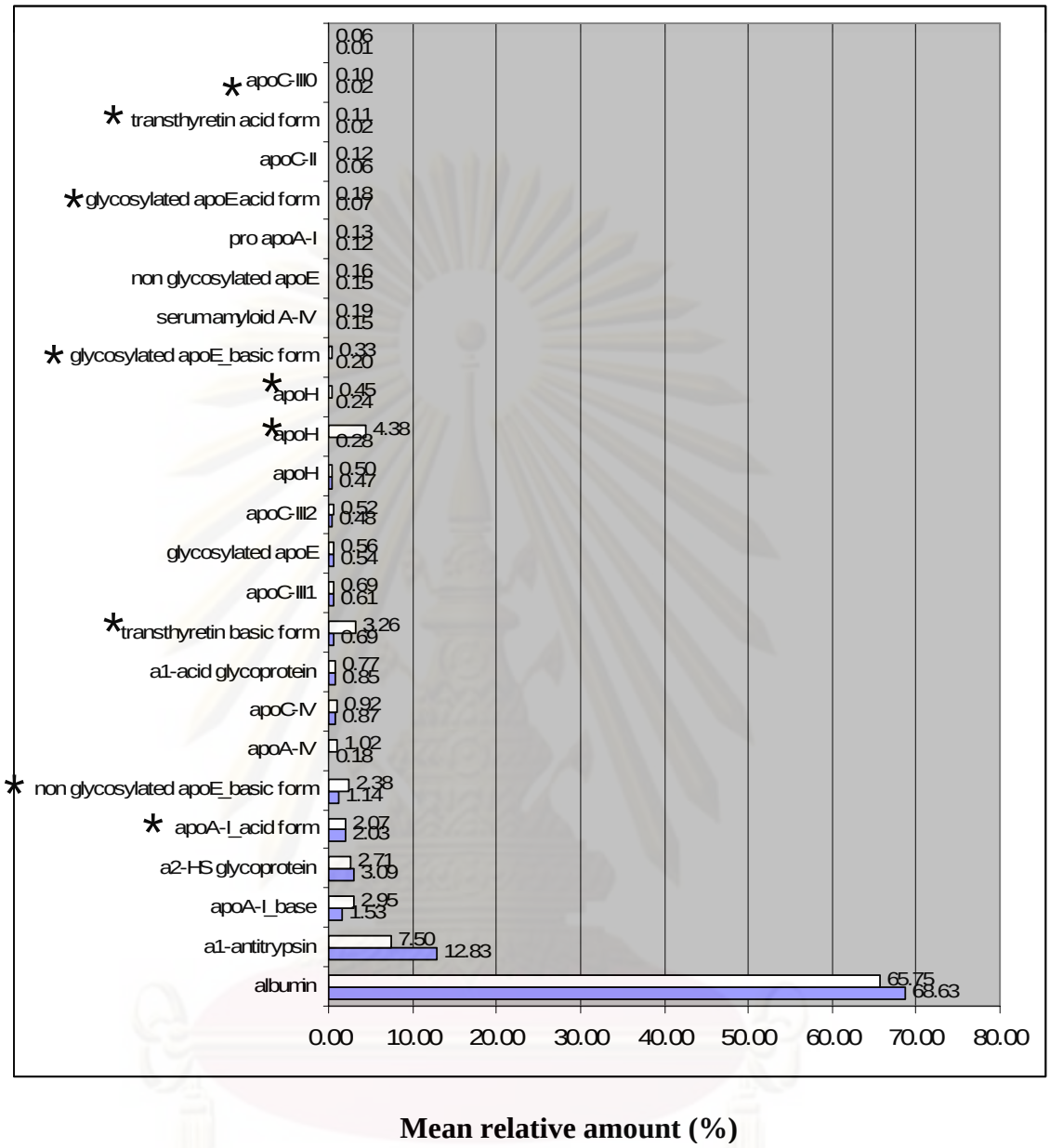


Figure 4.24 Quantitative differences in VLDL-associated proteins between postprandial state (white bar) and fasting state (black bar) from HIR group were calculated from the mean relative amount (%) of each protein. ***** $P<0.05$ compared with fasting state.

The relative amounts of apoC-III_0, transthyretin both acid and basic isoforms, apoA-I acid isoform, apoH, non glycosylated apoE basic isoform, glycosylated apoE basic isoform, glycosylated apoE acid isoform were significantly more in postprandial state.

Table 4.20 The alteration of VLDL proteins from fasting to postprandial state in higher insulin resistance diabetes (HIR) group.

Proteins	Increase	Decrease
albumin		↓
α1-antitrypsin		↓
apoA-I_base	↑	
α2-HS glycoprotein		↓
apoA-I_acid form	↑	
non glycosylated apoE basic form	↑	
apoA-IV	↑	
apoC-IV	↑	
α1-acid glycoprotein		↓
transthyretin basic form	↑	
apoC-III_1	↑	
glycosylated apoE	↑	
apoC-III_2	↑	
apoH	↑	
apoH	↑	
apoH	↑	
apoE_base	↑	
serum amyloid A-IV	↑	
non glycosylated apoE	↑	
pro apoA-I	↑	
glycosylated apoE acid form	↑	
apoC-II	↑	
transthyretin acid form	↑	
apoC-III_0	↑	
non glycosylated apoE acid form	↑	

CHAPTER V

DISCUSSION

5.1 Experiment I: Study on the influence of insulin resistance on diabetic lipoprotein profiling including small dense LDL particles size.

In this study, we observed the postprandial lipid alteration response to a high fat meal in ten non-diabetic subjects as a controls group and eleven type2 diabetic patients who moderated metabolic control (HbA1c<8%) with optimal fasting triacylglycerol (TAG< 150 mg/dl) and total cholesterol (TC< 240 mg/dl) (Table 4.2). No patient was receiving insulin treatment or lipid-lowering therapy. The diabetes were classified into two groups according to homeostasis model assessment of insulin resistance or HOMA-IR as lower insulin resistance, LIR (HOMA-IR<2.5) and higher insulin resistance, HIR (HOMA-IR 2.5-5.0). All three groups were comparable for age, waist-to-hip ratio, fasting total cholesterol and triacylglycerol level (Table 4.1).

Insulin resistance plays the important role in the dyslipidemia such as hypertriacylglycerolemia, predominance of small dense LDLs and low level of HDLs which considered as a common feature of diabetic. However, both groups of our diabetic subjects had the level of fasting TAG concentration less than 150 mg/dl with different insulin resistance. Although diabetic subjects had the fasting TAG level in normal range and was not statistically significant difference from non-diabetic subjects but it was highly correlated to HOMA index ($r = 0.888$, $p<0.01$) (Figure 4.2) and this relationship also found between HOMA index and postprandial TAG as well (Figure 4.1). However, these relationships were not found in control. This clearly shown insulin resistance plays the role in regulation of both fasting TAG and postprandial hypertriacylglycerol in diabetes. This result was agreed with the study of Annuzzi *et al.*, 2004 who studied the role of insulin resistance in development of dyslipidemia in type2 diabetic patients who had optimal blood glucose and normal fasting triacylglycerol. The contrast result was the increasing of postprandial triacylglycerol in control seems to not differ from LIR diabetic group, because this study we analyzed lipid profile at one point-four hours postprandial whereas previous

study followed lipid profile over 4-6 hours and used postprandial incremental area to analyze data. Total cholesterol before and after the test meal were similar among the three groups, this result similar to the study of Takayanagi *et al*, 2004 who study the postprandial state in Japanese type2 diabetes.

Insulin level in both LIR and HIR diabetic subject groups after postprandial state more increased than control group with significant differences ($p<0.05$) between control and diabetic groups which observed from the increasing of insulin from before and after meal in percent (Table 4.3). The postprandial glucose level still higher than control (Table 4.2), this is the result of insulin resistance or insulin failed to regulte glucose level. In addition, this study also showed an influence of insulin resistance on the risk of coronary artery disease by predominance of small dense LDL particles size and lower HDL concentration which found only in higher insulin resistance diabetic group despite of they had a fasting normal level triacylglycerol. Postprandial dyslipidemia especially TAG level was considered as a more powerful risk factor for cardiovascular disease than fasting TAG in all subjects which considered from the significant correlation between postprandial triacylglycerol and postprandial small dense LDL particles size in all subjects (Figure 4.6). This result was agreed with the study of Kondo *et al.,* 2001 which TAG level correlated negatively with the predominant LDL size both in non diabetic and type2 diabetic subjects and also the study of Koba *et al.,* 2003 which studied in coronary heart disease (CHD) patients, found the postprandial TAG contribute the small dense LDL formation.

Concerning the type of lipid abnormality induced by the meal, this study shows the increasing of postprandial triacylglycerol was mainly in chylomicrons and VLDL and more increasing in HDL fraction of HIR diabetic group compared to other groups. Healthy people secrete increased insulin in postprandial state to reduce the secretion of VLDL by liver. While, in type2 diabetes either fasting normal triacylglycerol level or the increasing in VLDL fraction during the postprandial state is explained by a reduced inhibitory effect of insulin on VLDL secretion and increasing in large VLDL (Rivellese *et al*, 2004). Together with reducing of lipoprotein lipase (LPL) activity in diabetes (Goldberg 20001) and increase in hepatic lipase (HL) in postprandial state found in type2 diabetic patient and in states of insulin

resistance (Chen *et al*, 1993) leading to reduce in HDL level as found in our HIR diabetic group. The significantly increasing of postprandial triacylglycerol in HDL of HIR diabetic group could be due to a higher activity of cholesterol ester transfer protein in the diabetic patients (Taskinen, 2003). However, these dyslipidemia were not found in LIR diabetic group this may be due to this group trend to be insulin deficiency rather than be insulin resistance which insulin level was lower than control although it did not significantly difference at $p<0.05$.

5.2 Experiment II: Study on the influence of insulin resistance on the alteration of apolipoproteins of very low density lipoproteins (VLDL) between diabetes mellitus type2 and non-diabetic subjects in postprandial state.

5.2.1 Optimum Conditions for Analysis of VLDL Associated Proteins Using Two-Dimensional Electrophoresis

Postprandial hypertriacyglycerolemia is a common phenomenon in type2 diabetes even with normal fasting TAG level and this hyperlipidemia occurred from the high level of large VLDL particle (Rivellese *et al.*, 2004). VLDL mainly composes with triacylglycerol and same as other lipoproteins, VLDL still compose with phospholipids, cholesterol and apoproteins. Apoproteins play important roles in the lipoprotein metabolism, both regulation of metabolism and acting as a ligand for lipoprotein receptors. Thus, the alteration of apoproteins affects the lipoprotein metabolism. To study the influence of insulin resistance on protein profiling of VLDL, proteomics tools composed with two-dimensional electrophoresis (2-DE) and mass spectrometry was used.

VLDL fraction was prepared using sequential ultracentrifugation, delipidated, and pellet proteins were resuspended in rehydration buffer before analyzed with 2-DE and mass spectrometry. Protein profiling of diabetic VLDL in both fasting and postprandial state were compared to those of non diabetic subjects.

The sample preparation step is an important step in proteomics study base on 2-DE using immobiline drystrip and SDS-PAGE. Since the limitation of protein could

be loaded in first dimension, IEF. The abundant protein in plasma such as albumin restricts the amount of others protein loaded into strip. Here, we performed two centrifugation steps at same condition to reduce the contamination of albumin. In previous study, Mancone and college analyzed human VLDL proteome using 2-DE and MALDI-TOF/TOF (Mancone *et al*., 2007). They used two steps centrifugation to reduced the contamination of albumin before performed 2-DE, but they used density gradient ultracentrifugation whereas we used sequential ultracentrifugation. However, contaminated albumin was also reduced.

As we known, VLDL composes with apoproteins, mainly apoB, apoE and apoC and their isoforms. However, one of the challenges in this study is how to separate apoC-II and apoC-III and their isoforms from each other in homogenous PAGE, because their molecular weights are almost the same. Previous studies by Karlsson *et al.*, 2005 and Mancone *et al*., 2007 used 12%T SDS-PAGE to analyze VLDL and LDLs associated proteins using 2-DE technique. First, we decides to use 12%T SDS-PAGE to analyze VLDL proteins. The result was the same as Mancone *et al*. apoC-II, apoC-III and their isoforms migrated together to the dye front. So, the 12.5%T and 13%T SDS-PAGE were used instead. However in 12.5%T gel apoC-III migrated closer to dye front than 13%T SDS-PAGE and whether protein migrate more than apoC-III might lose (figure 4.11). In addition to %T in SDS-PAGE, the electric current used for running gel also affects separated resolution as well. Although the higher current lead to shorter run time but the separated resolution was decreased.

5.2.2 VLDL Protein Profiling Using 2D-PAGE and Mass Spectrometry

Six pools of VLDL fraction were obtained from three group of control, LIR, HIR subjects both fasting and postprandial plasma. After protein were resolved by 2-DE, gels were stained with colloidal coomassie blue and identified by mass spectrometry. Several proteins that have been identified as VLDL proteins including their isoforms were identified as shown in figure 4.12 and table 4.10. The results presented apoA-I, apoA-IV, apoB-100, apoC-II, apoC-III, apoC-IV, apoE, apoH,

albumin, α-1-antitrypsin, α-2-HS-glycoprotein, α-1-acid-glycoprotein serum amyloid A-IV protein and transthyretin which is a novel protein found in VLDL.

ApoA-I is biosynthesized as a 267-residue precursor protein, preproapoA-I, which undergoes co-translational cleavage. The resulting proapoA-I contains a hexapeptide attached to the amino-terminus of mature apoA-I which is secreted into plasma and lymph and undergoes extracellular post-translational cleavage to the mature 243-residue apoA-I (Sprecher *et al*, 1984). Mature apoA-I is a polymorphic protein which can be identified by two-dimensional electrophoresis.

In this study, we found 3 isoforms of apoA-I, spot number 3a-3c which the spot pattern was the same pattern as in human plasma. ApoA-I major isoform was spot number 3b, de-amidated apoA-I isoform was spot 3a and proapoA-I was spot 3c. In the apoA-I mass spectrum, the presence of 2201.1117 and 1611.7781 mass peaks corresponding to amino acid 84-101 and 70-83, respectively represent apoA-I was unmodified with glycosylation both Thr^{78} and Thr^{92} (Karlsson *et al.*, 2005). In addition, the de-amidated apoA-I changes a net charge to more acidic position in 3a compared to 3b, which fits with their positions in the 2-DE pattern. Compare to the study of VLDL proteins using proteomics tool by Mancone *et al*., 2007 we can identify more apoA-I isoforms than them. They found only one isoform whereas we found three isoforms. The study of human plasma apolipoproteins using 2-DE by Sprecher *et al*., 1984 showed apoA-I have four isoforms.

ApoA-IV is a 46-kDa glycoprotein of 376-residue and associated with nascent chylomicrons, HDL and in a small portion with VLDL and LDL (Green *et al*., 1980). ApoA-IV is required for the efficient apoC-II mediated activation of lipoprotein lipase. We found only one isoform of apoA-IV (spot 4) in our VLDL samples. This result was the same as the study of Mancone *et al.* and Karlsson *et al.*

ApoB-100 is a 513 kDa protein of 4536-residue and is mainly apoproteins in LDL and VLDL. ApoB-100 is unique among the apolipoproteins in having a relatively high β-sheet content (~20%). Unlike other apolipoproteins, apoB-100 does not transfer among lipoprotein particles. In this study we detected apoB-100 in the top

region of SDS-PAGE because the %T we used in the second dimension not permit protein molecular weight more than 250 kDa to penetrate in.

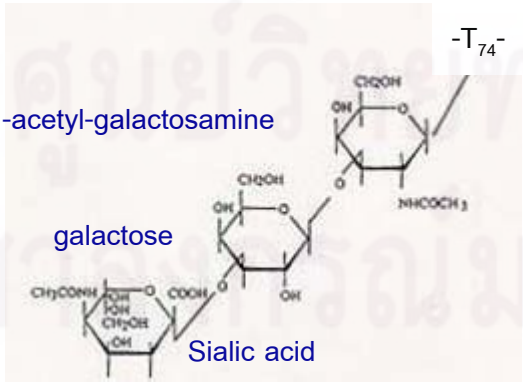
ApoC-II is synthesized as a 101-residue precursor, preapoC-II, which undergoes co-translational cleavage to mature apoC-II which contains 79 amino acids. In this study, we found only one isoform of apoC-II (spot 6) and also agree with the study of Karlsson *et al*., 2005. While Mancone *et al*. 2007 could not separate apoC-II from apoC-III isoforms.

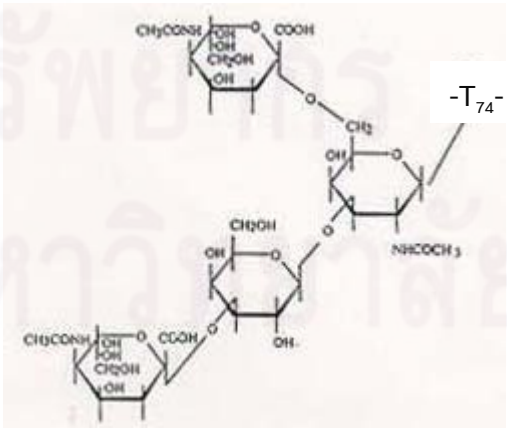
ApoC-III is synthesized as a 99-residue precursor, preapoC-III, and cleaved to mature apoC-III through co-translational cleavage. Mature apoC-III contains 79 amino acids and is polymorphic. ApoC-III occurs in human plasma in three isoforms depending on the number of post-translational sialyl groups added. The sugar moiety of apoC-III consists of 1 molecule of galactose, 1 molecule of N-acetyl-galactosamine, and 1, or 2 molecules of sialic acid for apoC-III_1 and apoC-III_2, respectively. ApoC-III_0 was not added any at Thr^{74}. The amino acid sequence of apoC-III and sugar chains make different isoform of apoC-III were shown below.

ApoC-III_0

SEAEDASLLS FMQGYMKHAT KTAKDALSSV QESQVAQQAR GWVTDGFSSL KDYWSTVKDK FSEFWDLDPE VRPT_{74}SAVAA

Sugar chain (apoC-$III_{1,2}$)





ApoC-III_1 (one sialic acid) **ApoC-III_2** (two sialic acid)

We detected three polymorphism of apoC-III, spots 5a-5c which 5a and 5b are probably post-translation sialyl groups added to Thr^{74}, a common modification of apoC-III (Bondarenko *et al*., 1999) lead to more molecular mass and acidic position. The unmodified apoC-III detected in spot 5c.

ApoC-IV has only been described in humans by Kotite *et al.,* 2003. The result suggest that apoC-IV has a role in the regulation of lipoprotein–lipid metabolism: two polymorphisms in the human apoC4 gene, Leu36Pro and Leu96Arg, have been found to be associated with plasma TG levels. ApoC-IV gene encoded a protein of 127 residues with a typical signal peptide of 27 residues and a predicted molecular mass of 11020 Da. We detected one isoforms of apoC-IV, *pI* 9.5 (spot 8) with the presence of mass peak 1330.7278 represents 54-64 amino acid position show this isoform corresponds to unglycosylated form from four isoforms of *N*-glycosylation described by Kotite *et al*.

ApoE is a 299-amino acids glycoprotein and synthesized as preapoE, amino acid precursor protein. PreapoE further co-translational cleavage of the 18-residue prepeptide to mature apoE and secreted into plasma as a sialylated apoprotein. However, the predominant apoE isoforms in plasma contain no sialic acid group.

The common isoforms of apoE are different at two positions in the amino acid sequence, positions 130 and 176. Three isoforms of apoE designed as shown in table.

apoE isoform	**amino acid residue at position 130**	**amino acid reidue at position 176**
apoE2	Cys	Cys
apoE3	Cys	Arg
apoE4	Arg	Arg

Six phenotypes are present in population: E2/2, E3/3, E4/4, E2/3, E2/4, and E3/4. The most common is E3/3 which considered being normal and the E2/2 phenotype is type III hyperlipoproteinemia which the most common mutation is the conversion of Arg 176 to Cys 176.

In this study, we found 6 isoforms, spots numbers 1a-1f. From MS/MS sequencing of all spots represented apoE revealed our subjects are the apoE3 homozygous donor from the presence of the 1221.51, 1185.63 and 1103.6199 mass peaks which correspond to R/LGADMEDVC^{130}GR/L, R/LLRDADDLQK/R^{176} and K/R^{176}LAVYQAGAR/R, respectively. Because apoE2 has cysteine residues at both positions 130 and 176 while apoE4 has two arginine residues at those positions. The differences in *pI* and MW of apoE spots occur from the modifications. Although we could not identify the modification, the previous study by Mancone *et al*., 2007 demonstrated that one of the modifications was glycosylation at Thr^{212} and it was possible that another post-translational modification might occur.

ApoH also known as P2-glycoprotein I, is a 50 kDa plasma protein that has associated with lipoprotein metabolism. ApoH is a polymorphic protein and is found in three isoforms. It is present in plasma and is found in the two major lipoprotein fractions, VLDL and HDL. Its ability to activate lipoprotein lipase suggests that it plays a part in triglyceride metabolism and directly moved TAG within, out of, between cells. We found three isoforms of apoH (spot 2a-2c). This result was contrast to previous studies by Mancone *et al*., 2007 who also used 2-DE to study the VLDL associated proteins. They did not found any isoform of apoH.

Serum amyloid A-IV is acute phase protein preferentially associated to HDL and found in other lipid particles such as LDL. ApoA-IV is required for efficient release of apoC-II from either HDL or VLDL, which then allows for LPL-mediated hydrolysis of TG in nascent chylomicrons (Goldberg *et al*., 1990). In HDL, six isoforms of serum amyloid A-IV were found, whereas two isoforms were found in LDL (Karlsson *et al*., 2005). We found only one isoform of serum amyloid A-IV in our VLDL sample.

Transthyretin (TTR) is a plasma protein of four identical subunits of 14 kDa that is mainly synthesized by the liver and the choroid plexus of the brain. TTR functions as a carrier for both thyroxine (T4) and retinol (vitamin A). A fraction of plasma TTR circulates in HDL through binding to apoA-I (Sousa *et al.*, 2000). This study, we found two isoforms of TTR (spot 14a and 14b) in VLDL which this is a novel finding.

5.2.3 VLDL Protein Profiling of Type2 Diabetes Mellitus

The lipoprotein profiling results of non diabetes and type2 diabetes varied with insulin resistance showed the dyslipidemia especially in postprandial state was the result of insulin resistance. However, it is not known whether insulin resistance is associated with changes in the protein composition of lipoprotein particles particular in VLDL. Present study, proteomics tool was used and an alteration of protein profile in VLDL from diabetic subjects compared to non diabetic subjects was found.

Both fasting state and postprandial state, several VLDL proteins from diabetic groups ware changed to more or less amount compared with non diabetic group.

Transthyretin which has not been identified in VLDL before was significantly higher amount in both diabetic group than control from postprandial state, $p<0.05$ (figure 4.18 and 4.19) and significantly higher only in HIR from fasting state, $p<0.05$ (figure 4.21). The unique predominance of transthyretin in postprandial HIR diabetic group was confirmed from significantly increase in postprandial than fasting state (figure 4.24), which only found in HIR. Transthyretin was found in HDL and recently in LDL which significantly more abundant in obese subjects than lean subjects (Karlsson *et al.*, 2009). Transthyretin was considered to correlate with insulin resistance, from the study of Mody *et al*., 2008 found transthyretin elevated in plasma from insulin-resistant obese mice. Also transthyretin act as a protease which degraded apoA-I, this may affect HDL biology and the development of atherosclerosis by reducing cholesterol efflux and increasing of apoA-I amyloid deposits which are frequently found in the aortic intima (Liz *et al*., 2004). This might explain the effect of transthyretin on apoA-I level in our diabetic subjects.

ApoA-I major isoform was significantly lower in both diabetic groups compared with non diabetic subjects, $p<0.05$ both fasting and postprandial state, apoA-I acid isoform was significantly less in both diabetic groups than control in fasting state and higher than control in postprandial state (figure 4.18 to 4.21). The increasing of apoA-I acid isoform in postprandial state were significantly differ with $p<0.05$ than fasting in all subject groups (figure 4.22 to 4.24). The increasing of

apoA-I acid isoform in postprandial state represents direct intestinal biosynthesis, and that the chylomicrons and VLDL are secreted into the lymph with an increased content of apoA-I acid isoform. Alternatively, apoA-I acid isoform may have a high affinity for triglyceride-rich lipoprotein particles, and an increase in concentration of these lipoproteins during fat absorption could result in an increase in apoA-I acid isoform content in these lipoproteins by transfer from a pre-existing pool of apoA-I acid isoform (Ghiselli et al., 1983). ApoA-I itself has anti-oxidant and anti-inflammatory properties (Danky *et al.*, 1999), thus decreased concentration of apoA-I correlated to increase risk of CAD.

ApoC-III was found higher amount in LIR than control and significantly higher in HIR diabetic than control in fasting state (figure 4.20 and 21) and more significantly increase in both diabetic group than control in postprandial state (figure 4.18 and 19). ApoC-III plays important role in triglyceride metabolism by inhibits lipoprotein lipase. The apoC-III gene is down regulated by thyroid hormone, insulin and cytokine as interleukin-1, thus apoC-III increase production in insulin resistant state (Cohn *et al.*, 2004). The high amount of apoC-III is considered to relate with cardiovascular disease. The significant alteration of apoC-III_0 relative level from fasting to postprandial state in all subject groups were found (figure 4.22 to 4.24). However, this alteration had little or no impact on lipoprotein metabolism because the glycosylation of apoC-III is not an absolute prerequisite for its secretion and ability to associate with plasma lipoproteins (Jong et al., 1999).

Contrast with apoC-III, apoE was found significantly higher in control than LIR and HIR groups in postprandial state (figure 4.18 and 19) and significantly lower in HIR group in fasting state, $p<0.05$ (figure 4.20 and 21). ApoE is also considered important in the metabolism of triacylglycerol-rich lipoprotens, apoE in VLDL functions as ligand for the VLDL receptors. Both increasing of apoC-III and decreasing of apoE have been demonstrated in LDL from patients with metabolic syndrome and type2 diabetes (Davidsson *et al*., 2005). The significantly increased of glycosylated apoE in postprandial state correlated with the increasing of triacylglycerol level in parallel (Grunfeld *et al*., 1997).

In case apoA-IV which required for the efficient apoC-II mediated activation of lipoprotein lipase and facilitated CETP. Fasting apoA-IV was found significantly lower amount in both diabetic groups than control especially in HIR group with $p<0.05$ (figure 4.20 and 21). On the other hand, apoA-IV levels in diabetic groups were lower than control in postprandial state (figure 4.18 and 19). The decreasing of apoA-IV level in higher insulin resistance diabetic subjects or HIR was agreed with Verges showed type2 diabetes had low apoA-IV levels and associated with insulin resistance because the apoA-IV levels are increased when type2 diabetes was treated with insulin (Verges, 1995). ApoA-IV is associated with increased prevalence of vascular disease.

ApoC-II was found significantly less in LIR and HIR than controls both fasting and postprandial state, $p<0.05$. The most decreasing of apoC-II was found in HIR. ApoC-II function as the activator of lipoprotein lipase, thus the decreasing of apoC-II correlated to VLDL catabolism.

Noteworthy, only apoH in postprandial HIR had the higher level than non diabetes (4.38 vs. 0.19 % mean relative amount) although it didn't significant difference (figure 4.19). However, the relative amount of apoH was significantly more in postprandial than fasting, which found only in HIR. ApoH levels were closely associated with TAG level and found higher in diabetic subjects (Ruiu et al., 1997 and Castro *et al.,* 2010). This could explain why apoH was not significantly increase in control, whereas LIR had much lower TAG than HIR group.

The alteration of apoproteins in both diabetic groups affect lipoprotein metabolism and lead to the complicated consequences in diabetes such as atherosclerosis. The decreasing of apoA-IV for efficient release of LPL and apoC-II for activate LPL activity whereas increasing of apoC-III inhibit LPL activity together with the decreasing of apoE which used for binding hepatic VLDL receptor, especially in postprandial state lead to the decreasing of VLDL catabolism and VLDL clearance leading to prolong VLDL circulation in blood, resulting to postprandial dyslipidemia in diabetes. Moreover the increasing of apoA-IV associated with

increased prevalence of vascular disease lead to increase the risk of developing CAD in diabetes.

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CHAPTER VI

CONCLUSIONS

This study demonstrates that insulin resistance associated with fasting and postprandial hypertriacylglycerol in plasma of type2 diabetes. In addition, insulin resistance also associated with the increasing of triacylglycerol in lipoprotein as well and the most increasing of triacylglycerol was found in very-low density lipoprotein (VLDL). The increasing of triacylglycerol in chylomicron, VLDL, high-density lipoprotein (HDL) was found significantly difference with $p<0.05$ in diabetic with higher insulin resistance (HIR) compare to non diabetic and lower insulin resistant diabetic subjects (LIR) who have HOMA-IR similar to non diabetic subjects. Moreover, HIR who had the most increasing of triacylglycerol in postprandial state had the smallest particles size of small dense LDL and lower HDL cholesterol than other groups, which this correlation was also found in hypertriacylglycerolemic (Taskinen, 2005) and myocardial infarction patients (Tsunoda *et al.*, 2004).

Because of the overproduction of VLDL and hypertriacylglycerolemia in type2 diabetes was considered as a result of insulin resistance (Ginsberg, 2000). The alterations of proteins lead to alter VLDL fate in metabolism. Thus, protein profiling of VLDL and the influence of insulin resistance on VLDL proteins was study using proteomics tools, two-dimensional electrophoresis and mass spectrometry.

From the analysis of protein profiling of VLDL, 26 spots represents 14 different proteins were identified including their isoforms as shown in table below.

Spot No.	Proteins
1a-1f	apoprotein E with six isoforms; • non glycosylated apoE 3 isoforms • glycosylated apoE 3 isoforms
2a-2c	apoprotein H with three isoforms
3a-3c	apoprotein A-I with three isoforms • pro apoA-I • apoA-I acid isoform • apoA-I major soform
4	apoprotein A-IV
5a-5c	apoprotein C-III with three isoforms • apoC-III_0 • apoC-III_1 • apoC-III_2
6	apoprotein C-II
7	serum amyloid A-IV protein
8	apoprotein C-IV
9	α-1-antitrypsin
10	α-2-HS-glycoprotein
11	α-1-acid-glycoprotein 1
12	albumin
13	apoprotein B-100
14a-14b	Transthyretin

Moreover, by mass spectrometric techniques, the isoforms and methylated modification of apoE could be identified. Although the role of the methylated modification on apoE was not fully understood but it was confirmed that mass spectrometry could be used for the detection of protein modification though the molecular mass increased only 14 Da.

The proteomics study of VLDL protein profiling from type2 diabetes found the enrichment of transthyretin in VLDL from HIR diabetic group. To our knowledge, transthyretin have not been identified in VLDL before, particular in diabetic VLDL. Moreover, we found some distinct alterations in the VLDL protein contents e.g., apoA-I major isoform, apoA-I acid isoform, apoA-IV, apoE, apoC-II, apoC-III, apoH and novel protein transthyretin occur in diabetic subjects compare to non diabetic subjects.

Proteomics technique base on 2D-PAGE and mass spectrometry which deeply analyze to isoforms and modifications of proteins was proved to be the powerful technique for identified, discovered novel protein and opened new possibilities for protein expression profiling in diabetic disorder.

However, the present study is a pilot study, and the further investigation with a larger number of subjects is required to establish this finding. This study demonstrated that protein profiling of lipoprotein particles can be used to obtain important information that cannot be found from lipid profiling assessments or single biomarker analysis in plasma.

The limitations in these studies arise from the volume of plasma obtained from subjects and complicated methodology. The method we used for separated VLDL can not separate VLDL sub fractions. The 2-DE staining method that we used was colloidal coomassie blue with sensitivity lower than fluorescence staining-sypro ruby, so higher amount of proteins was needed including VLDL compose only 8-10% protein and our subjects were not hypertriacylglycerolemia especially in fasting state. So the amount of VLDL proteins we obtained from individual subject is not enough for 2-DE. The groups are small in numbers of subjects, selection bias must be considered as well. Since the groups were only selected from the well lipid controlled diabetes.

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APPENDICES

APPENDIX A

คณะกรรมการพิจารณาจริยธรรมการวิจัยในคน กลุ่มสหสถาบัน ชุดที่ 1 จุฬาลงกรณ์มหาวิทยาลัย
วิทยาลัยวิทยาศาสตร์สาธารณสุข จุฬาฯ อาคารสถาบัน 2 ชั้น 4 ซอยจุฬาลงกรณ์ 62 ถนนพญาไท
เขตปทุมวัน กรุงเทพฯ 10330 โทรศัพท์: 0-2218-8147 โทรสาร: 0-2253-2395 E-mail: irbcu@yahoo.com

COA No. 014/2551

เอกสารรับรองโครงการวิจัย

โครงการวิจัย : อิทธิพลของภาวะไตรเอกลีเซอรอลสูงในเลือดหลังมื้ออาหารต่อการเปลี่ยนแปลงของอะโปโปรตีนบนไลโปโปรตีนชนิดที่มีไตรเอกลีเซอรอลเป็นองค์ประกอบหลัก ในผู้ป่วยเบาหวานชนิดที่ 2 ที่มีภาวะดื้อต่ออินซูลินต่างกันโดยแมสสเปกโทรเมทรี

ผู้วิจัยหลัก : นางสาวศิริพร แสงสุธรรม นิสิตหลักสูตรวิทยาศาสตรดุษฎีบัณฑิต

หน่วยงาน : สหสาขาวิชาชีวเวชศาสตร์ บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย

คณะกรรมการพิจารณาจริยธรรมการวิจัยในคน กลุ่มสหสถาบัน ชุดที่ 1 จุฬาลงกรณ์มหาวิทยาลัย อนุมัติในแง่จริยธรรมให้ดำเนินการศึกษาวิจัยเรื่องดังกล่าวได้

ลงนาม.....
(ศาสตราจารย์ นายแพทย์ปรีดา ทัศนประดิษฐ์)
ประธาน

ลงนาม.....
(ผู้ช่วยศาสตราจารย์ ดร.นันทรี ชัยชนะวงศาโรจน์)
กรรมการและเลขานุการ

วันที่รับรอง : 25 มกราคม 2551 **วันหมดอายุ** : 24 มกราคม 2552

เงื่อนไข

1. หากใบรับรองหมดอายุ การดำเนินการวิจัยต้องยุติ เมื่อต้องการต่ออายุต้องขออนุมัติใหม่ล่วงหน้าไม่ต่ำกว่า 1 เดือน เงื่อนไข
2. ต้องดำเนินการวิจัยตามที่ระบุไว้ในโครงการวิจัยอย่างเคร่งครัด
3. ใช้เอกสารข้อมูลสำหรับอาสาสมัคร/ประชากรตัวอย่าง, ใบยินยอม, และเอกสารเชิญเข้าร่วมวิจัย (ถ้ามี) เฉพาะที่ประทับตราของคณะกรรมการฯ เท่านั้น แล้วส่งสำเนาใบแรกที่ใช้ เอกสารดังกล่าวมาที่คณะกรรมการฯ
4. หากเกิดเหตุการณ์ไม่พึงประสงค์ร้ายแรง ต้องรายงานคณะกรรมการฯ ภายใน 5 วันทำการ
5. หากมีการเปลี่ยนแปลงการดำเนินการวิจัย ให้ส่งคณะกรรมการฯ พิจารณารับรองก่อนดำเนินการ
6. **โครงการวิจัยไม่เกิน 1 ปี ส่งบทคัดย่อผลการวิจัยในรูป CD ภายใน 30 วัน เมื่อโครงการวิจัยเสร็จสิ้น**
7. **โครงการวิจัยเกิน 1 ปี ส่งรายงานความก้าวหน้าการวิจัยทุกปี เมื่อโครงการวิจัยเสร็จสิ้นแล้ว ส่งบทคัดย่อผลการวิจัยในรูป CD ภายใน 60 วัน**

APPENDIX B

เทพธารินทร์ Theptarin Hospital
3850 ถนนพระราม 4 คลองเตย กรุงเทพฯ 10110
3850 Rama 4 Road, Klongtoey, Bangkok 10110
Tel. +66 2 348 7000 Fax +66 2 249 8774
www.theptarin.com

Documentary Proof of Ethics Committee on Researches Involving Human Subjects.

Theptarin Hospital

No. 2/2008

Title of Project : The influence of postprandial hypertriacylglycerolemic state type 2 diabetes with varied insulin resistance on alterations of apoproteins in triacylglycerol-rich lipoproteins study by mass spectrometry

Protocol Number : ID 02-2008

Principle Investigator : Ms.Siriporn Sangsuthum

Name of Department : Doctor of Philosophy Program in Biomedical Sciences
Graduate School, Chulalongkorn University

Document Reviewed : Protocol Document

The aforementioned documents have been reviewed and acknowledged by Ethics Committee on Researches Involving Human Subjects, based on the Declaration of Helsinki.

Signature of Chairman
Ethics Committee on Researches
Involving Human Subjects

Somboon Vongterapak,MD

Date of Approval June 13, 2008

Excellence
Teamwork
Hospitality
Integrity
Continuous Improvement
Social Responsibility

APPENDIX C

Table C1. Amino acids and their Masses organized according to molecular weight.

Amino acid	Letter code (3 letters)	Letter code (1 letter)	Mass
Glycine	Gly	G	57
Alanine	Ala	A	71
Serine	Ser	S	87
Proline	Pro	P	97
Valine	Val	V	99
Threonine	Thr	T	101
Cysteine	Cys	C	103
Isoleucine	Ile	I	113
Leucine	Leu	L	113
Asparagine	Asn	N	114
Aspartic acid	Asp	D	115
Glutamine	Gln	Q	128
Lysine	Lys	K	128
Glutamic acid	Glu	E	129
Methionine	Met	M	131
Histidine	His	H	137
Phenylalanine	Phe	F	147
Arginine	Arg	R	156
Tyrosine	Tyr	Y	163
Tryptophan	Trp	W	186

APPENDIX D

Table D1. VLDL proteins identified with MS/MS ions search

Spot No.	Protein	Accession No.	*pI*	Mass (da)	Masses matched	Amino acid position	Sequence coverage
1a	ApoE	P02649	5.65	36132	1624.7944	20-33	64%
					1246.5691	34-43	
					2072.0188	34-50	
					2729.3872	57-79	
					2092.0324	91-108	
					1729.8370	94-108	
					1221.5118	122-132	
					1646.7934	138-152	
					1901.9629	138-154	
					1185.6353	166-175	
					1103.6199	176-185	
					1496.7947	210-224	
					2295.1720	210-231	
					1413.7463	247-258	
					1312.7099	259-269	
					1113.5778	261-269	
					1032.5352	270-278	
					1776.8869	279-292	
					1535.7079	281-292	
					1619.7904	301-317	
1b	ApoE	P02649	5.65	36132	1246.5691	34-43	59%
					2072.0188	34-50	
					2729.3872	57-79	
					2092.0324	91-108	
					1729.8370	94-108	
					1221.5118	122-132	

Table D1. Continued

Spot No.	Protein	Accession No.	*pI*	Mass (da)	Masses matched	Amino acid position	Sequence coverage
					1646.7934	138-152	
					1901.9629	138-154	
					1496.7947	210-224	
					1312.7099	259-269	
					1113.5778	261-269	
					1032.5352	270-278	
					1776.8869	279-292	
					1535.7079	281-292	
					2531.2769	293-317	
					1619.7904	301-317	
1c	ApoE	P02649	5.65	36132	1624.7944	20-33	64%
					1246.5691	34-43	
					2072.0188	34-50	
					2729.3872	57-79	
					2092.0324	91-108	
					1729.8370	94-108	
					1221.5118	122-132	
					1646.7934	138-152	
					1901.9629	138-154	
					1185.6353	166-175	
					1103.6199	176-185	
					1496.7947	210-224	
					2295.1720	210-231	
					1413.7463	247-258	
					1312.7099	259-269	
					1113.5778	261-269	
					1032.5352	270-278	
					1776.8869	279-292	

Table D1. Continued

Spot No.	Protein	Accession No.	*pI*	Mass (da)	Masses matched	Amino acid position	Sequence coverage
					1535.7079	281-292	
					1619.7904	301-317	
1d	apoE	P02649	5.65	36132	1246.5691	34-43	55%
					2072.0188	34-50	
					2729.3872	57-79	
					1307.6465	80-90	
					2092.0324	91-108	
					1729.8370	94-108	
					1221.5118	122-132	
					2306.1689	133-152	
					1646.7934	138-152	
					1901.9629	138-154	
					1496.7947	210-224	
					1312.7099	259-269	
					1032.5352	270-278	
					1776.8869	279-292	
					1535.7079	281-292	
					2531.2769	293-317	
					1619.7904	301-317	
1e	apoE	P02649	5.65	36132	1624.7944	20-33	52%
					1246.5691	34-43	
					2072.0188	34-50	
					2729.3872	57-79	
					2092.0324	91-108	
					1729.8370	94-108	
					1237.5067	122-132	
					2306.1689	133-152	

Table D1. Continued

Spot No.	Protein	Accession No.	*pI*	Mass (da)	Masses matched	Amino acid position	Sequence coverage
					1496.7947	210-224	
					1312.7099	259-269	
					1032.5352	270-278	
					1776.8869	279-292	
					1535.7079	281-292	
					2531.2769	293-317	
					1619.7904	301-317	
2a	ApoH	P02749	8.34	38273	1912.9969	22-38	44%
					2382.0838	39-58	
					1900.0645	63-78	
					1771.9695	64-78	
					1501.7711	83-96	
					3522.7350	124-154	
					2818.4550	130-154	
					2628.1108	228-250	
					2384.9889	230-250	
					1638.7647	288-301	
					2212.9980	306-324	
					2084.9031	307-324	
2b	ApoH	P02749	8.34	38273	1912.9969	22-38	31%
					1900.0645	63-78	
					1771.9695	64-78	
					2818.4550	130-154	
					2628.1108	228-250	
					2384.9889	230-250	
					1021.5193	271-279	
					2212.9980	306-324	

Table D1. Continued

Spot No.	Protein	Accession No.	*pI*	Mass (da)	Masses matched	Amino acid position	Sequence coverage
2c	ApoH	P02749	8.34	38273	1912.9969	22-38	37%
					2382.0838	39-58	
					1771.9695	64-78	
					2818.4550	130-154	
					2384.9889	230-250	
					1638.7647	288-301	
					2212.9980	306-324	
3a	ApoA-I	P02647	5.56	30759	1461.8443	35-47	70%
					1234.6809	37-47	
					1649.8854	37-51	
					1814.8435	48-64	
					1399.6620	52-64	
					1611.7781	70-83	
					2201.1117	84-101	
					1931.9265	86-101	
					2617.2660	86-107	
					1251.7454	121-130	
					1410.6601	131-140	
					1282.5652	132-140	
					1466.7841	143-155	
					1151.6298	156-164	
					1030.5117	165-173	
					1300.6411	185-195	
					1584.8008	185-197	
					1156.6200	202-212	
					1214.6143	220-230	
					1011.5713	231-239	
					2224.3582	231-250	

Table D1. Continued

Spot No.	Protein	Accession No.	*pI*	Mass (da)	Masses matched	Amino acid position	Sequence coverage
					1229.7020	240-250	
					1385.7078	251-262	
					1513.8028	251-263	
3b	ApoA-I	P02647	5.56	30759	1461.8443	35-47	65%
					1234.6809	37-47	
					1649.8854	37-51	
					1814.8435	48-64	
					1399.6620	52-64	
					1611.7781	70-83	
					2201.1117	84-101	
					1931.9265	86-101	
					2617.2660	86-107	
					1334.6387	108-118	
					1251.6136	121-130	
					1379.7085	121-131	
					1282.5652	132-140	
					1030.5117	165-173	
					1300.6411	185-195	
					1584.8008	185-197	
					1156.6200	202-212	
					1214.6143	220-230	
					1011.5713	231-239	
					1229.7020	240-250	
					1385.7078	251-262	
					1513.8028	251-263	

Table D1. Continued

Spot No.	Protein	Accession No.	*pI*	Mass (da)	Masses matched	Amino acid position	Sequence coverage
4	ApoA-IV	P06727	5.28	45371	1633.8311	52-65	58%
					1406.7041	66-78	
					1534.7991	66-79	
					1438.7932	79-90	
					1343.6932	100-110	
					1234.6670	113-123	
					1103.5611	135-143	
					1286.6579	144-154	
					2361.2400	167-187	
					1992.9864	170-187	
					1299.6783	190-200	
					1082.5284	201-209	
					1309.6918	201-211	
					1101.5666	212-220	
					1349.6463	222-233	
					2889.4331	222-246	
					1557.7973	234-246	
					1926.9435	288-304	
					2083.0446	288-305	
					1351.6520	306-316	
					1195.5509	307-316	
					1214.6441	317-326	
					2061.0643	327-345	
					1804.9108	329-345	
					2189.1229	329-348	
					1318.6558	349-359	

Table D1. Continued

Spot No.	Protein	Accession No.	*pI*	Mass (da)	Masses matched	Amino acid position	Sequence coverage
5a	apoC-III	P02656	5.23	10845	2016.0236	42-60	37%
					1715.8438	45-60	
					1195.5874	61-71	
					2075.0000	61-78	
5b	apoC-III	P02656	5.23	10845	1715.8438	45-60	34%
					1195.5874	61-71	
					2075.0000	61-78	
5c	apoC-III	P02656	5.23	10845	1715.8438	45-60	34%
					1195.5874	61-71	
					2075.0000	61-78	
6	apoC-II	P02655	4.72	11277	1285.5826	42-52	59%
					1036.5189	53-61	
					1034.5284	62-70	
					1303.7136	62-72	
					2838.4361	73-98	
					2232.1348	78-98	
					2547.2414	78-101	
7	SAA4 (Serum amyloid A-IV)	P35542	9.27	14797	1640.7981	23-37	63%
					1131.5343	27-37	
					1847.8373	38-52	
					1716.8332	58-74	
					2866.4389	79-103	
					1204.5472	104-113	

Table D1. Continued

Spot No.	Protein	Accession No.	*pI*	Mass (da)	Masses matched	Amino acid position	Sequence coverage
8	apoC-IV	P55056	9.19	14543	1330.7278	54-64	35%
					1955.9067	65-79	
					1544.6718	80-91	
					2269.0838	80-98	
9	α-1-antitrypsin	Q86U18	5.37	46707	1640.8562	50-63	30%
					2056.9378	161-178	
					2089.0884	199-225	
					1890.8483	226-241	
					1802.9526	284-298	
					1077.5203	299-306	
					1109.5968	315-324	
					1014.6073	325-334	
					1832.9156	335-352	
10	α-2-HS-glycoprotein	P02765	5.43	39300	1195.6197	58-67	8%
					2080.0160	318-337	
11	α-1-acid-glycoprotein1	P02763	4.93	23497	1159.5815	43-51	30%
					1751.9471	109-123	
					1444.6544	127-138	
					1707.8468	139-153	
					1111.5186	171-179	
					1239.6136	171-180	

Table D1. Continued

Spot No.	Protein	Accession No.	*pI*	Mass (da)	Masses matched	Amino acid position	Sequence coverage
12	Albumin	Q9P157	5.92	69321	1225.5979	35-44	19%
					1148.6077	66-75	
					1073.5353	206-214	
					1622.7833	348-360	
					1466.8358	361-372	
					2044.0881	397-413	
					1638.9305	438-452	
					1510.8355	439-452	
					1127.6914	549-558	
					1341.6275	570-581	
					1140.6866	598-609	
13	apoB-100	Q13787	6.61	515241	2189.1588	76-93	34%
					1138.5295	101-110	
					2209.1670	140-157	
					1665.9189	159-174	
					2019.9280	197-214	
					2543.2360	230-252	
					2021.0503	276-293	
					1700.8634	440-454	
					1887.9434	491-507	
					1518.7898	513-525	
					1722.9305	577-590	
					2610.3653	591-613	
					1270.6769	642-654	
					1745.8988	655-669	
					2153.1256	675-695	
					1307.6761	696-707	
					2052.1255	779-797	

Table D1. Continued

Spot No.	Protein	Accession No.	*pI*	Mass (da)	Masses matched	Amino acid position	Sequence coverage
					1553.8599	805-818	
					2823.4290	1001-1024	
					2563.2840	1056-1078	
					2087.9980	1101-1118	
					1237.6455	1135-1145	
					2027.0357	1146-1164	
					1612.7443	1189-1202	
					1094.5067	1216-1224	
					1274.7057	1230-1240	
					2072.0538	1241-1259	
					2515.2569	1260-1280	
					1305.6969	1334-1344	
					2497.1609	1393-1415	
					2532.2319	1451-1474	
					2127.0583	1537-1556	
					1504.7733	1647-1661	
					1475.6094	1738-1750	
					2374.2744	1782-1802	
					1221.5989	1803-1813	
					3272.5408	1868-1897	
					1601.8202	1917-1930	
					1600.8461	1968-1982	
					2405.1135	1983-2002	
					2163.9345	1985-2002	
					2407.0564	1985-2004	
					1086.6033	2003-2012	
					1398.7970	2013-2025	
					3415.9251	2013-2043	
					1819.9720	2044-2095	

Table D1. Continued

Spot No.	Protein	Accession No.	*pI*	Mass (da)	Masses matched	Amino acid position	Sequence coverage
					2625.2500	2063-2083	
					1140.6040	2101-2109	
					2434.2029	2113-2133	
					1993.9282	2118-2133	
					1557.7886	2151-2164	
					1453.8391	2196-2208	
					1968.0105	2225-2240	
					1579.7478	2241-2255	
					1590.8617	2296-2309	
					1065.5818	2310-2318	
					1607.7865	2348-2360	
					1027.6026	2377-2385	
					1964.0942	2450-2467	
					1536.8035	2475-2488	
					2338.2718	2489-2509	
					1927.8952	2560-2575	
					1429.7817	2579-2591	
					1946.0547	2592-2609	
					1832.9673	2610-2625	
					1044.5968	2626-2634	
					2960.4670	2645-2669	
					2448.2285	2797-2818	
					1865.8618	2837-2853	
					1359.6994	2877-2888	
					2927.3825	2936-2962	
					1797.8897	2970-2989	
					2528.2183	3035-3057	
					2825.4137	3068-3092	
					1355.8064	3137-3148	

Table D1. Continued

Spot No.	Protein	Accession No.	*pI*	Mass (da)	Masses matched	Amino acid position	Sequence coverage
					2261.2347	3137-3155	
					2156.1300	3184-3202	
					3562.8099	3245-3276	
					1564.7919	3277-3291	
					2242.3089	3292-3311	
					1890.9462	3481-3497	
					2290.1580	3481-3501	
					2460.2285	3538-3558	
					2031.9498	3639-3656	
					1321.6666	3713-3724	
					2970.4984	3737-3762	
					1615.8821	3776-3790	
					2465.3669	3847-3868	
					2299.1696	4056-4076	
					1398.6416	4133-4145	
					2608.3133	4146-4168	
					2365.1914	4148-4168	
					3380.7001	4148-4177	
					1197.5522	4214-4222	
					1308.6925	4223-4234	
					1704.8570	4447-4461	
					1889.9734	4447-4463	
14a	transthyretin	P02766	5.52	15877	1365.7517	42-54	65%
					1521.7100	55-68	
					1393.6150	56-68	
					2454.1438	69-90	
					2450.1979	101-123	
					2515.3322	124-146	
					2488.2737	125-147	

Table D1. Continued

Spot No.	Protein	Accession No.	*pI*	Mass (da)	Masses matched	Amino acid position	Sequence coverage
14b	transthyretin	P02766	5.52	15877	1521.7100	55-68	59%
					1393.6150	56-68	
					2454.1438	69-90	
					2450.1979	101-123	
					2488.2737	125-147	

Table D2. VLDL proteins identified with peptide mass fingerprinting

Spot No.	Protein	Accession No.	*pI*	Mass (da)	Masses matched	Amino acid position	Sequence coverage
1f	apoE	P02649	5.65	36132	1246.5691	34-43	18%
					844.2740	44-50	
					899.4245	51-60	
					1729.8370	94-108	
					948.1677	177-185	
					1497.8773	210-224	
					817.5856	225-231	
					1313.6799	259-269	
3c	apoA-I	P02647	5.50	30759	1234.6809	37-47	43%
					1399.6620	52-64	
					1931.9265	86-101	
					1251.6136	121-130	
					1282.5652	132-140	
					873.2431	148-155	
					1030.5117	165-173	
					781.2178	178-184	
					1300.6411	185-195	
					1011.5713	231-239	

BIOGRAPHY

Miss Siriporn Sangsuthum was born on 25 April 2515 at Bangkok. She graduated with bachelor degree of science, Department of General Science, Faculty of Science, Chulalongkorn University in 2538 and graduated with master degree of science in biotechnology, Program of Biotechnology, Faculty of Science, Chulalongkorn University in 2546. She received the grant from the Ministry of Education to study in doctoral degree in Biomedical Sciences, Graduate School, Chulalongkorn University in 2546. Now, she is a scientist at Department of Clinical Chemistry, Faculty of Allied Health Sciences, Chulalongkorn University.