CHAPTER 2

MATERIALS AND METHODS

2.1 Study design

Preparation of LDL subfractions by density gradient ultracentrifugation was performed using the method developed by Guerin *et al*. 1996 [29]. This technique classifies LDL into two major fractions including sdLDL and bdLDL. Concentration of protein and lipid in each LDL subfraction were determined based on enzymatic and Lowry methods. Proteins from each fraction were separated with 1, 2 dimensional gel electrophoresis. After electrophoresis, gels were silver stained. Proteins were excised from gels, digested with trypsin and then subjected to liquid chromatography linked directly to tandem mass spectrometry (LC/ESI-Ion trap MS/MS). Proteins were identified using the database search engine Mascot.

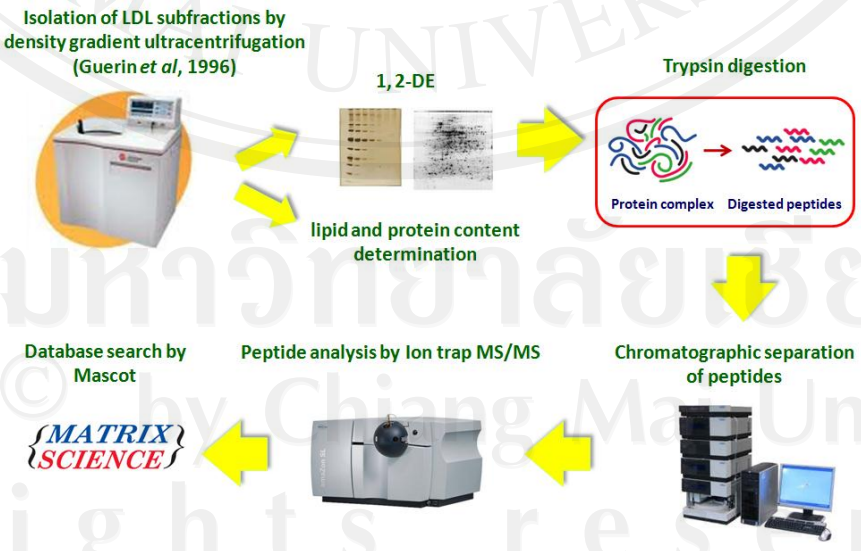

Figure 18 Schematic overview of study design

2.2 Sample preparation

Blood samples in EDTA-containing tubes were obtained from 4 healthy volunteers after an overnight fast. Preparation of LDL subfractions by density gradient ultracentrifugation was performed by a method described by Guerin *et al* [(29)]. Plasma (3 mL) was adjusted to 1.21 kg/L by adding of KBr. A density gradient was then constructed as follows: 2 mL of a NaCl/KBr solution (*d*=1.24 g/mL), 3 mL plasma (*d*=1.21 g/mL), 2 mL of a solution (*d*=1.063 g/mL), 2.5 mL of a solution (*d*=1.019 g/mL), and 2.5 mL of a NaCl solution (*d*=1.006 g/mL). After centrifugation in a Beckman SW41 Ti rotor at 40,000 rpm for 44 hours at 15^{o}C, solutions were collected from the top of the tube in 12 fractions corresponding to

VLDL (*d*<1.017 g/mL), IDL (*d*=1.018 to 1.019 g/mL), LDL-1 (*d*=1.019 to 1.023 g/mL), LDL-2 (*d*=1.023 to 1.0239 g/mL), LDL-3 (*d*=1.029 to 1.039 g/mL), LDL-4 (*d*=1.039 to 1.050 g/mL), LDL-5 (*d*=1.050 to 1.063 g/mL), HDL_{2b} (*d*=1.063 to 1.091 g/mL), HDL_{2a} (*d*=1.091 to 1.110 g/mL), HDL_{3a} (*d*=1.110 to 1.133 g/mL), HDL_{3b} (*d*=1.133 to 1.156 g/mL), and HDL_{3c} (*d*=1.156 to 1.179 g/mL). Each subfraction was PBS buffer (pH 7.2) exchange for 3 days. This technique classifies LDL into different fractions, including small, dense LDL (LDL-4, 5) and bouyant LDL (LDL-1-3).

2.3 Lipid and protein content determination

2.3.1 Total cholesterol determination

The method for total cholesterol determination is based on an enzymatic colorimetric method using a CHOD-PAP system to measure the cholesterol during the hydrolysis of a CE by cholesterol esterase. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase.

Table 5 Experimental design for total cholesterol determination

Reagents	**Blank**	**Standard**	**Sample**
Cholesterol reagent (ml)	0.5	0.5	0.5
Standard (µl)	-	10	-
Sample (µl)	-	-	10
Incubate at room temperature for 10 minute			
Double distilled water (ml)	0.5	0.5	0.5
Read absorbance at 500 nm			

2.3.2 Triglycerides determination

The method for triglycerides determination is also based on an enzymatic colorimetric method (PAP). The triglycerides in the sample were hydrolyzed to glycerol and fatty acids by lipase. Glycerol was then phosphorylated by glycerol kinase (GK) in the presence of ATP. In the next step, glycerol-3-P was oxidized by glycerol-3-P oxidase (GPO) in the presence of molecular oxygen (O_2). A colored product was formed from hydrogen peroxide, 4-aminoantipyrine in the presence of the peroxidase (POD).

Table 6 Experimental design for triglycerides determination

Reagents	**Blank**	**Standard**	**Sample**
Triglycerides reagent (ml)	0.5	0.5	0.5
Standard (µl)	-	10	-
Sample (µl)	-	-	10
Incubate at room temperature for 10 minutes			
Double distilled water (ml)	0.5	0.5	0.5
Read absorbance at 500 nm			

2.3.3 Protein determination

2.3.3.1 BCA assay

Protein concentration in the LDL subfractions was determined via the BCA assay. This assay relies on reduction of cupric ions (Cu^{2+}) to cuprous ions (Cu^{1+}) by protein in alkaline solution. Colorimetric detection is based on the formation of complexes between bicinchoninic acid (BCA) and Cu^{1+}. This purple product exhibits a strong absorbance at 562 nm [60]. Determination of the protein concentration of samples is done by comparing their absorbance values against the standard curve.

Table 7 Experimental design for protein determination using BCA assay

Reagents	**Standard**	**Sample**
Standard (µl)	10	-
Sample (µl)	-	10
Working reagent (µl)	190	190
Incubate at 37°C for 30 minutes		
Read absorbance at 562 nm		

2.3.3.1 Lowry assay

For 1 and 2-DE, the Lowry protein assay was used for determination of protein concentration. The principle is based on the interaction of Cu^{2+} and protein in alkaline solution, resulting in reduction of Cu^{2+} to Cu^{1+}. The Cu^{1+} can be detected with Folin-Ciocalteu phenol reagent (phosphomolybdic-phosphotungstic acid). Cu^{1+} reduction of Folin-Ciocalteu phenol reagent produces a blue color that can be read at 750 nm. The amount of color produced is proportional to the amount of protein.

Table 8 Experimental design for protein determination using the Lowry assay

	Blk	**Std 2 µg/µl**	**Std 4 µg/µl**	**Std 6 µg/µl**	**Std 8 µg/µl**	**Std 10 µg/µl**	**Sample**
0.5% SDS /0.15% DOC (µl)	5	4	3	2	1	-	-
2 mg/ml BSA (µl)	-	1	2	3	4	5	-
Sample (µl)	-	-	-	-	-	-	5
Reagent A (µl)	200	200	200	200	200	200	200
Incubate at room temperature for 30 minutes in the dark							
Reagent B (µl)	50	50	50	50	50	50	50
Incubate at room temperature for 30 minutes in the dark							
Read absorbance at 750 nm							

2.4 One-dimensional gel electrophoresis (1-DE)

Excess salts and lipids were removed using a clean-up kit. Samples were transferred into a 1.5 ml microcentrifuge tube. 600 µl of precipitant was added and incubated on ice for 15 min. Then, 300 µl of coprecipitant was added and centrifuged at 12,000 g for 5 min. Supernatant was carefully removed without disturbing the pellet. Next, 80 µl of coprecipitant was added, incubated on ice for 5 min and centrifuged at 12,000 g for 5 min. Supernatant was removed without disturbing the pellet. 25 µl of de-ionized water was added and vortexd for 10 s. Then, 1 ml of wash buffer (pre-chilled for at least 1 h at -20^{o}C) and 5 µl of wash additive was added. Samples were vortexed until pellet is fully dispersed and incubated on ice for 30 min. Next, samples were vortexed for 30 s every 10 min and centrifuged at 12,000 g for 5 min. Supernatant was discard and pellet was allowed to dry about 5 min. Pellet was resuspended in 0.5% SDS solution and stored at -20^{0}C.

Protein concentration was determined by the Lowry method. Samples were mixed with 5X SDS loading buffer (0.125 M Tris-HCl pH 6.8, 20% glycerol, 5% SDS, 0.2M DTT, 0.02% bromphenol blue) and heated for 5 min in boiling water. Each of the LDL subfractions containing 5 µg proteins were then loaded onto the 12.5% SDS-polyacrylamide gel. Molecular weight protein standard marker was applied on the gel. SDS-PAGE was performed using miniPAGE AE-6530 until the tracking dye reached the bottom of the gel.

2.5 Two-dimensional gel electrophoresis (2-DE)

Protein delipidation, precipitation and solubilization were performed as follows. Briefly, 14 ml of ice-cold tri-butylphosphate:acetone:methanol (1:12:1) were added to 1 ml of each LDL subfraction and incubated at 4^{o}C for 90 min. The precipitates were pelleted by centrifugation (2800×g for 15 min at 4^{o}C), washed sequentially with 1 ml of tri-butylphosphate, acetone, methanol and then air dried. Precipitates were resuspended in lysis solution (7 M urea, 2 M thiourea, 4% CHAPS, 10 mM Pefabloc protease inhibitor) and stored at -20^{o}C. Isoelectric focusing was performed using a strip holder.

Isoelectric focusing

Rehydration solutions containing 300 µg protein and 18 mM DTT were prepared. Then, 1% IPG buffer pH 3-10 Linear was added and incubated for 45 min in the dark. Solutions were centrifuged at 10,000 rpm for 15 min and rehydration solutions containing 300 µg proteins were applied into the strip holder. Next, the 18 cm, pH 3-10 L IPG strip were carefully placed with the gel-side down. The strips were overlay with Immobiline DryStrip Cover Fluid. Then, the cover on the strip holder was placed and strips were allowed to rehydrate. The rehydration period can be set at the first step of program.

Table 9 Running conditions for 18 cm, pH 3-10 L IPG strips

Step Voltage mode	Voltage (V)	kVh
Step and Hold	500	0.5
Gradient	1000	6.0
Gradient	8000	13
Step and Hold	8000	12.2

IPG strips were used immediately for the second-dimensional analysis or stored at -70^{o}C. Equilibration was performed prior to the second dimension.

Equilibration

The strips were placed in individual tubes. Then, DTT containing equilibration buffer (100 mg per 10 ml) was added and incubated for 15 min. Buffer was poured off and IAA containing equilibration buffer (250 mg per 10 ml) was added. Next, solutions were incubated for 15 min, poured off and rinsed with SDS buffer.

SDS-PAGE

The strips were placed onto 12.5% SDS gels. Then, warm agarose solution was slowly pipetted onto the IPG strip. MW marker was applied next to the basic end of the strips. Electrophoresis was performed at 400 mA, 600 V, 10 Watt for about 4 hours.

2.6 Staining

After electrophoresis, gels were silver stained. First, gels were fixed in 50% methanol/12% acetic acid overnight, then washed with 35% ethanol twice for 5 min. Gels were then sensitized with 0.02% sodium thiosulfate for 2 min and washed with deionized water. Gels were then stained in 0.2% silver nitrate for 20 min, washed with water and developed with 6% sodium carbonate/sodium thiosulfate until dark protein bands were visible. Finally, staining reaction was stopped with Na-EDTA. After staining, gels were captured by scanning and stored in TIFF formats

2.7 Tryptic digestion

Proteins were excised from the gels and cut into small pieces (1 mm^3) using sterile surgical blades with aseptic technique and transferred to 96 well plates. The gel pieces were subjected to in-gel digestion using a method developed by Genome Institute, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Thailand. The gel pieces were washed with 200 µl of sterile water for 5 min and then dehydrated twice with 200 µl of 100% acetonitrile (ACN) for 5 min. Fifty milliliters of reduction buffer (10 mM dithithreitol in 10 mM NH_4HCO_3) were added and incubated for 1 hour. Then, an alkylation buffer (100 mM iodoacetamide/10 mM NH_4HCO_3) was added and incubated in the dark for 1 hour. The gel pieces were dehydrated twice with 200 µl of 100% ACN for 5 min. Proteins were digested by adding 20 µl of trypsin (10 ng/µl in 50% ACN/10 mM NH_4HCO_3), followed by incubation for 20 min. Gel pieces were then mixed with 30% ACN and let stand overnight at room temperature. The supernatants were transferred to separated wells and the peptides were further

extracted from the gel pieces by incubation in 50% ACN/0.1% FA for 10 minutes at room temperature twice. The supernatants from the two steps were then pooled and dried in a hot air oven. The peptides were stored at -80^{o}C for further mass spectrometric analysis.

2.8 LC-MS/MS analysis

Peptides in 96-well plate were resuspended in 15 µl of 0.1% FA, then transferred to 1.5 ml microcentrifuge tubes, mixed, and centrifuged (10,000 ×g, 5 min). About 15 µl of the peptide solution was applied into 0.25 ml insert conical bottom vial and analyzed by LC/ESI-ion trap MS/MS. The peptides were analyzed using an UltiMate 3000 LC System (Dionex Ltd., U.K.) couple to HCT Ultra PTM Discovery System (Bruker Daltonics Ltd., U.K.). Peptides were separated on a nanocolumn (Acclaim PepMap 100 C18, 3µm, 100A, 75 µm id × 150 mm). Eluent A was 0.1% formic acid and eluent B was 80% ACN in water containing 0.1% formic acid.

2.9 Protein quantification and identification

Protein quantification and normalization was performed with Decyder MS 2.0 Differential Analysis Software (DeCyderMS, GE Healthcare) based on peptide ions signal intensities. For identification of proteins, the analyzed data were subjected to the database search via Mascot software (Matrix Science, London, UK) against the NCBI database. Comparison of protein intensities was performed using MultiExperiment Viewer (MeV) software. Student's t-test was used to determine the statistical differences with *P*-value less than 0.05