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Modified low-density lipoprotein from *Mycobacterium tuberculosis* induces foamy macrophage formation

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Abstract of the Thesis

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Tuberculosis (TB) is one of the major causes of mortality in the world, causing approximately 1.5 million deaths every year, among which children take a great part. However, due to the perception that children are rarely infectious or they rarely develop severe disease, children with TB have always been neglected. What is worse, diagnosis of childhood TB is difficult, making it harder to control the disease. Foamy macrophages are a key component in the pathology of TB, and the formation of foamy macrophages foresees the progression of infection to active disease. Low-density lipoprotein (LDL) is one of the major lipoproteins that contain cholesterol, and important metabolism of cholesterol enables bacterial survival. After modification in the host, LDL could lead to foamy macrophage formation in the tubercular granuloma. We hypothesized that the modified LDL in patients with TB which induces foamy macrophage formation is different from other forms of LDL. Therefore, modified LDL in patients with TB could serve as a biomarker for TB diagnosis. Our long-term objective is to characterize modified LDL in TB

patients and investigate its function in foamy macrophage formation. In this work, we first prepare *Mtb*-modified LDL, and then we set up macrophage experiment to investigate its function towards foamy macrophage formation. Then we focus on the characterization of *Mtb*-modified LDL using agarose gel electrophoresis, Western blot analysis, and MALDI-TOF analysis.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

Acro-LDL	Acrolein-conjugated low-density lipoprotein
ApoB	Apolipoprotein B
BSA	Bovine serum albumin
SR-AI and II	Class A scavenger receptors type I and II
VD_3	1,25-dihydroxyvitamin D_3
DTT	Dithiothreitol
FBS	Fetal bovine serum
HRP	Horseradish peroxidase
HIV	Human immunodeficiency virus
HNE	4-hydroxynonenal
IAA	Iodoacetamide
LOX-1	Lectin-like oxidized LDL receptor-1
LDL	Low-density lipoprotein
MDA	Malondialdehyde
Mtb	*Mycobacterium tuberculosis*
Mtb-LDL	*Mtb*-modified LDL
FDP-Lys	N^{ε}-(3-formyl-3, 4-dehydropiperidino)lysine
MP-Lys	N^{ε}-(3-methylpyridinium)lysine
OADC	Oleate-albumin-dextrose-NaCl-catalase
OxLDL	Oxidized low-density lipoprotein
PFA	Paraformaldehyde

PMA	Phorbol 12-myristate 13-acetate
PVDF	Polyvinylidene fluoride
Rt	Room temperature
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TFA	Trifluoroacetic acid
TLR-4	Toll-like receptor 4
TB	Tuberculosis
WHO	World Health Organization

Chapter 1 Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*Mtb*), is a major global health problem. It ranks as the second leading cause of death from infectious disease in the world, just after the human immunodeficiency virus (HIV). In the Global Tuberculosis Report 2014, the World Health Organization (WHO) estimated that 9.0 million people developed TB and 1.5 million died from the disease in 2013.[1] Due to the unacceptably high mortality of TB, the development of effective TB diagnosis is important.

The global burden of TB among children cannot be underestimated. In 2013, there were approximately 550,000 cases and 80,000 deaths among children (aged <15).[1] However, the diagnosis of TB in children is challenging. The most common method for diagnosing TB, which is targeting sputum smear-positive cases of TB for epidemic control, greatly ignores children, who rarely have sputum smear-positive TB.[2] Thus, a biomarker is urgently needed for childhood TB diagnosis.

When *Mtb* is inhaled by the host, it establishes an infection in lung macrophages. The infected macrophages trigger an inflammatory response leading to the recruitment of blood and tissue macrophages, lymphocytes, and dendritic cells to form a granuloma, the hallmark pathologic lesion associated with a TB infection. Foamy macrophages, a granuloma-specific cell population characterized by their high lipid content, are a key component in the pathology of TB. They constitute a nutrient-rich reservoir for *Mtb* persistence and also serve an active function that leads to lung cavitation and the release of infectious bacilli. One important reason for foamy macrophages formation is the uptake of modified low-density lipoprotein (LDL) by macrophages.

Therefore, we think it is important to identify the function and characterization of modified LDL in TB patients, which is a potential biomarker for TB diagnosis, especially for childhood TB diagnosis.

In this chapter, an overview of TB, current challenges of childhood TB diagnosis, foamy macrophages, and native LDL as well as modified LDL will be discussed.

1.1. *Mycobacterium tuberculosis*

Tuberculosis (TB) is an "old" disease, and its causative agent, the bacterium *Mycobacterium tuberculosis* (*Mtb*), is estimated to have originated about 15,000 to 20,000 years ago.[3] On March 24, 1882, Dr. Robert Koch discovered *Mtb*. At the time, one out of seven people living in the United States and Europe was killed by TB.[4] However, despite the development of modern medical technology, TB still remains one of the deadliest infectious diseases of humans.

Mtb could not only just lead to an asymptomatic latent infections but also cause an acute disease.[5] In individuals who are asymptomatic, *Mtb* stays in a dormant state for decades until the reactivated bacilli induce clinical disease.[6] Now, up to one-third of the human population are infected with the bacterium *Mtb*, among which 5-10% have a risk of developing active disease during their life.[7] However, until now, due to the similarity of the systemic immune response in those who have active disease and those who only have asymptomatic latent infection without active TB, no biomarker has been found for disease progression.[8]

1.2. Childhood tuberculosis and current challenges in diagnosis

Childhood tuberculosis, a leading cause of morbidity and mortality, is a serious problem, especially in developing countries.[9] Even if childhood tuberculosis accounts for only 6% of the global burden, it takes a larger proportion (15-20%) of all TB cases in developing countries.[9] Therefore, children with TB are important. Because children is the source where future cases will come from, ignoring childhood TB will lead to failure to control TB disease.[10]

However, people have always neglected children with TB. There are several reasons. In some degree, this is because children usually have paucibacillary disease, which means low bacteriologic yield.[2] This makes people think that they are not infectious, so they could not play significant role in propagation of the epidemic.[2] Also, only a few countries have prioritized child health organizations.[2] Another important reason is the difficulties in making an exact diagnosis of TB in children. Due to poor bacteriologic yields of childhood TB, the application of bacteriological confirmation by culture of *Mtb,* the accepted gold standard of TB diagnosis, is limited to a great extent.[11] TB in children is usually smear-negative. So sputum smear microscopy, the primary method for TB diagnosis in many developing countries, could only test out a small part of children (10-15%) with probable TB.[11] Also, while TB in adults is usually accompanied by specific symptoms and signs, symptoms of childhood TB are non-specific.[2] All these factors obstruct the accurate diagnosis of childhood TB.

1.3. The life cycle of *Mycobacterium tuberculosis*

Mtb, which are discharged as infectious droplets by people with active infection, are inhaled by individuals from the atmosphere.[12] These droplets remain airborne for a period of time, usually a couple of hours. The estimated minimum infectious dose is only a single bacterium, which makes transmission occur at an efficient speed.[12]

Infection is initiated after the inhalation of bacilli by host alveolar macrophages through phagocytosis.[8] Macrophages play an essential role as the first line of body's defense system in protecting the host against foreign substances.[13] When *Mtb* are inside macrophages, they replicate in phagosomes. By inhibiting phagosomes maturation and preventing phagosomes from fusion with acidic and hydrolytically active lysosomes, *Mtb* is able to avoid degradation in phagolysosomes.[14] In this way, *Mtb* could grow at an extremely rapid speed until the host has a protective immune response.[8]

The infected macrophages trigger a proinflammatory response leading to the recruitment of mononuclear cells from peripheral blood vessels.[15] Thus, the bacterial population further expand benefiting from having fresh host cells.[15] These mononuclear cells form the early stage of granuloma. The granuloma is a major histopathological feature of TB, and its function is the containment of *Mtb* at the site of primary infection to avoid bacterial spread to healthy tissues, and to centralize the immune response to a fixed infectious site.[16] In the early stage of granuloma formation, it consists of infected macrophages as its core with foamy macrophages and other monocytes around it, enclosed by lymphocytes.[8] As the granuloma matures, a fibrous capsule is developed, which extrudes most of the lymphocytes.[8] Also, in this stage, the macrophages

differentiate into different morphotypes, such as epithelioid cells, multinucleated giant cells, and foamy macrophages.[17]

The rapid bacterial replication ends after two to three weeks the emergence of lymphocytes.[15] At this stage, cells are recruited to the site of infection and the granuloma is fully vascularized.[15] Along with the disease progression, there is a decrease in the number of blood vessels penetrating the granuloma.[15] In the meantime, the number of foamy macrophages increases. Foamy macrophages are thought to be the major reason that lead progression to active disease, because they are presumed to be responsible for the necrosis in the granuloma center.[8] In immunocompetent patients with active disease, the infected tissues contain granulomas in all states of development from its formation to active disease, which means the progression is localized to granulomas individually, not systemically.[15] In the advanced stage, the center of the granuloma liquefies and the lung cavitates, releasing virulent bacilli into the airways.[8] This causes lung damage, resulting in the generation of aerosol with infectious *Mtb* when patients cough.[8] From being inhaled to being produced, the life cycle of *Mtb* is complete.

1.4. Differentiated macrophages

Differentiated macrophages are long-lived cells with specialized functions.[18] They originate from monocytes differentiation.[19] In order to mimic macrophage, several monocytic cell lines with different degrees of differentiation could be used.[20] There are different protocols to develop differentiated macrophages. The monocytic cell lines could be THP-1 cells, U937 cells, or HL-60 cells, and the stimuli that are commonly used to induce macrophage differentiation in

monocytic cell lines could be phorbol 12-myristate 13-acetate (PMA) or 1,25-dihydroxyvitamin D_3 (VD_3).[21] In terms of cell morphology and other factors, one study showed that THP-1 cells, activated with PMA and then rested by culture for a further 5 days without PMA, were similar to the phenotype of human monocyte-derived macrophages.[18] Therefore, in our experiment, we followed their protocol to induce differentiated macrophages.

1.5. Foamy macrophages

Many studies have found that it is the granuloma that determines the progression of TB from a latent infection to active disease.[8] The granuloma results from the interaction between *Mtb* and the host cells at the infection site.[8] In the granuloma, macrophages can differentiate into several different cell populations, and one of them is called foamy macrophages.

Foamy macrophages are macrophages filled with lipid-free vacuoles and lipid-rich bodies.[6] They could serve as a safe reservoir to prevent *Mtb* from direct contact with lymphocytes, and they could be a nutrient source for *Mtb* persisting in a dormant non-replicative state.[6] Electron microscopy has shown that bacilli-containing phagosomes were close to lipid bodies, and ultimately, bacilli were found in lipid bodies and had intracellular lipid inclusions, which suggested that *Mtb* could use lipid bodies as a nutrient source.[8] Also, one study has shown that foamy macrophages were located mainly at interface region surrounding the central necrotic area, and they were observed only in necrotic lessions.[6] All these phenomena suggested that foamy macrophages play an important role in the accumulation of caseous debris in the granuloma and the formation of necrosis.

1.6. Low-density lipoprotein

Foamy macrophages arise from the accumulation of esterified and non-esterified sterols as well as neutral lipids in lipid bodies.[22] The conversion from macrophages to foamy macrophages occurs via regulating uptake and release of low-density lipoprotein (LDL) particles from the serum.[8] Each LDL particle contains a single apolipoprotein B-100 (apoB100) molecule that has 4536 amino acid residues and a hydrophobic core comprising polyunsaturated fatty acid, varying numbers of triglycerides, and many esterified and non-esterified cholesterol molecules. This hydrophobic core is surrounded by a rim of phospholipids and non-esterified cholesterol.

LDL particles are endocytosed by macrophages through LDL receptors. The majority of the cholesterol in plasma lipoprotein exists as cholesteryl esters, which are processed in a two-compartment pathway.[23] After entering the macrophages, cholesteryl esters are transported to lysosomes, where they are hydrolyzed by lysosomal acid lipase.[23] Then the cholesterol goes through the lysosomal membrane and enters the cytoplasm, where it is re-esterified by Acyl-CoA cholesterol acyltransferase.[23] Finally, the re-esterified cholesterol is stored as cholesteryl ester lipid bodies in the cytoplasm.[23]

1.7. Modified low-density lipoprotein

Native LDL does not cause foamy macrophage formation. This is because not only differentiated macrophages have just a few LDL receptors but also the receptor is downregulated during cholesterol uptake, preventing the accumulation of excessive cholesterol.[24] Even when incubated with high concentrations of native LDL, very few macrophages convert to foamy

macrophages.[25] Therefore, there must be other mechanisms that can explain the accumulation of cholesterol in macrophages. Many studies have demonstrated that modification of LDL is a prerequisite for uptake by macrophages.[26] Scavenger receptors, which are specific macrophage receptors, are able to recognize modified LDL, and they are different from LDL receptors so they are not downregulated.[25] Modification of LDL occurs in arterial wall and in plasma circulation, and there are various mechanisms, such as oxidation, aggregation, and hydrolysis.[27] Then modified LDL is taken up into macrophages through a receptor-mediated endocytosis.[24] There are two well-known forms of modified LDL. One is oxidized LDL and the other is acrolein-conjugated LDL. They are known to induce foamy macrophages formation, especially in atherosclerosis.[28]

1.7.1. Oxidized low-density lipoprotein

The term oxidized LDL (OxLDL) refers to a variety of LDL preparations that have been oxidatively modified under certain conditions or isolated from biological sources.[29] There have been extensive studies on OxLDL over the last several decades, however, due to its heterogeneity, there is still no conclusion about the exact definition or composition of OxLDL. OxLDL can be broadly divided into four main categories: minimally oxidized LDL, mildly oxidized LDL, moderately oxidized LDL, and extensively oxidized LDL.[30] The degree of oxidation depends on the concentration of oxidizing agent and the time of oxidation.

Multiple receptors that belong to different classes are able to recognize different forms of OxLDL as well as mediate their cellular interactions. For example, class A scavenger receptors type I and II (SR-AI and II) are most specific for extensively oxidized LDL; class B scavenger

receptor CD36 can bind extensively and moderately oxidized LDL; and class E scavenger receptor lectin-like oxidized LDL receptor-1 (LOX-1) has a higher affinity to mildly-oxidized LDL.[29] As for minimally oxidized LDL, it is a very mildly oxidized form of LDL that is not sufficiently oxidized to be recognized by the scavenger receptors described above. Studies found minimally oxidized LDL is specifically recognized by a Toll-like receptor 4 (TLR-4).[31] Once bound to macrophage scavenger receptor, OxLDL is efficiently taken by macrophages, which leads to lipid accumulation.

LDL can be oxidized by metal ions, myeloperoxidase, lipoxygenases, and reactive nitrogen species.[32] In our study, we used the most common copper oxidation method for OxLDL preparation.

1.7.2. Acrolein-conjugated low-density lipoprotein

Lipid peroxidation products are characteristic of patients with advanced tuberculosis.[33] Aldehydes have been considered as the end products in lipid peroxidation. However, they can still react with biomolecules, including proteins and phospholipids.[34] Acrolein, a highly reactive unsaturated aldehyde, is the strongest electrophile among all α, β-unsaturated aldehydes.[35] It has high reactivity with nucleophiles, such as the sulfhydryl group of cysteine, amino group of lysine, or imidazole group of histidine.[35] In particular, acrolein derivatives N^{ε}-(3-formyl-3, 4-dehydropiperidino)lysine (FDP-Lys) and N^{ε}-(3-methylpyridinium)lysine (MP-Lys) have been detected in apolipoprotein B of native LDL in atherosclerosis (Figure 1-4).[36] This suggests that acrolein plays an important role on LDL modification. Recent studies showed that acrolein-

conjugated LDL (Acro-LDL) contributes to the development of atherosclerosis by inducing foamy macrophage formation.[28]

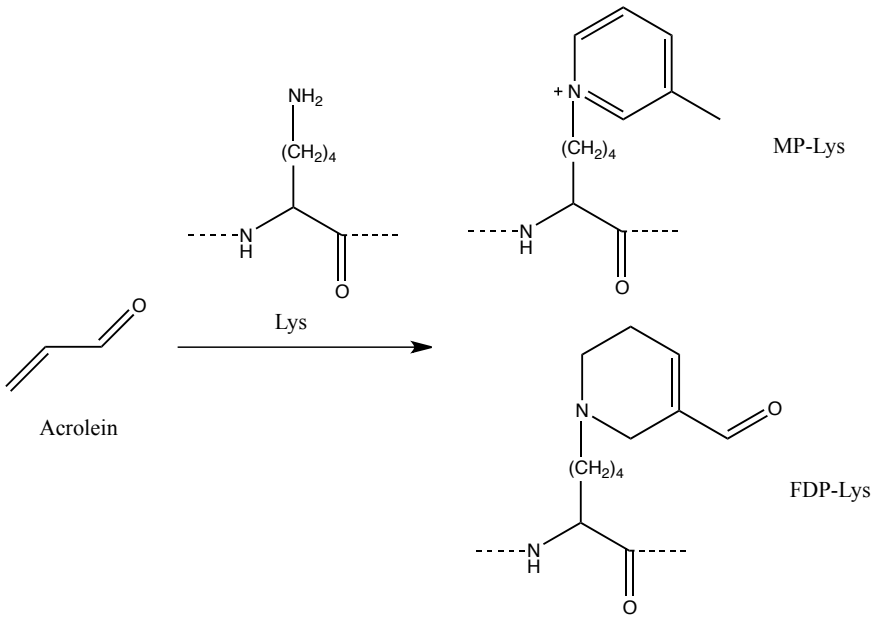


Figure 1-1. Acrolein reacts with Lys to form MP-Lys and FDP-Lys.

1.8. Characterization and function of *Mtb*-modified low-density lipoprotein

While a large number of studies have investigated the reason for cholesterol accumulation in atherosclerosis, there are many unknowns in TB. Our long-term goal is to characterize modified LDL in TB patients. In this study, we made *Mtb*-modified LDL *in vitro*, investigated its function in foamy macrophage formation, and tried to elucidate the *Mtb*-specific modification. Our first hypothesis was that LDL was modified when incubated with *Mtb* cells, and that *Mtb*-modified LDL was different from other forms of modified LDL, such as OxLDL and Acro-LDL. We also hypothesized that *Mtb*-modified LDL could induce foamy macrophage formation, an indispensable charactristics in the pathology of TB.

Our experimental strategy was to prepare *Mtb*-modified LDL, do macrophage experiments to investigate its function, and to use agarose gel electrophoresis, Western blot analysis, and MALDI-TOF analysis to determine its identity. In our experiments, intact LDL, OxLDL and Acro-LDL were used as controls.

Chapter 2 Experimental Section

2.1. Materials

THP-1 cells and Hep G2 cells were purchased from ATCC. RPMI medium 1640 (1X), MEM medium (1X), penicillin streptomycin (Pen Strep), phosphate buffered saline (PBS) (1X), HEPES (1M), and sodium pyruvate (100 mM) were purchased from Gibco. Fetal bovine serum (FBS) was purchased from Invitrogen. 2-mercaptoethanol, hematoxylin, Sudan Red 7B, barbital, bovine serum albumin (BSA), paraformaldehyde (PFA), iodoacetamide (IAA), dithiothreitol (DTT), potassium bromide, formic acid, phorbol 12-myristate 13-acetate (PMA), and copper sulfate were purchased from Sigma-Aldrich. Acrolein was purchased from Ultra Scientific. Oil Red O, anti-LDL (copper oxidized) antibody, and anti-LDL (MDA oxidized) antibody were purchased from Abcam. Agarose, acetonitrile, and ammonium bicarbonate were purchased from Fisher Scientific. Prestained protein ladder was purchased from New England BioLabs. PVDF membrane, goat anti-rabbit IgG (H+L)-horseradish peroxidase (HRP) conjugate, and immun-star HRP chemiluminescent substrate kit were purchased from Bio-Rad. LDL commercial and OxLDL commercial were purchased from Alfa Aesar. Anti-apoB antibody (H-300) was purchased from Santa Cruz. Goat anti-mouse IgG antibody, HRP conjugate was purchased from EMD Millipore.

2.2. Purification of low-density lipoprotein from Hep G2 cells

2.2.1. Cell culture

Hep G2 cells, a human liver cell line from hepatocellular carcinoma cells, were maintained in MEM medium with 10% heat-inactivated fetal bovine serum (FBS), 10 mM HEPES, 110 mg/mL sodium pyruvate, and 100 U/mL penicillin in an atmosphere of 5% CO_2/95% air. When the cells were grown to 80% confluence, low-density lipoprotein (LDL) particles as well as other proteins were isolated from culture supernatants by ultrafiltration through a 100-kDa molecular weight cutoff filter. Hep G2 growth medium was removed by concentrating the retentate and washing four times with PBS. Using the Bradford protein assay, protein concentrations of culture supernatants was measured, and the yield of LDL is about 2.4 mg protein/mL culture.

2.2.2. LDL isolation by ultracentrifugation

In order to remove albumin and other contaminants, LDL particles were separated by density gradient ultracentrifugation. Each 12 mL polyallomer open-top tube contained 7 mL sample in PBS, and potassium bromide was added and dissolved into each tube to adjust the density to 1.063 g/mL. The volume of the sample was slightly changed after adding salt into it, but we did not take that into account. After ultracentrifugation in Beckman SW 41 Ti rotor at 150,000 g for 48 h at 4 ºC, LDL particles were in the top 3-mL fraction. The top fraction was removed by pipetting. Then LDL particles were desalted by ultrafiltration through a 100-kDa molecular weight cutoff filter and washed four times with PBS. LDL particles were harvested and sterile filtered through 0.22 μm PVDF filters.

2.3. Preparation of modified LDL

2.3.1. Preparation of oxidized LDL

2 mg protein/mL of LDL in PBS was incubated with 50 μM $CuSO_4$ at 37 ºC for 24 h. In order to isolate oxidized LDL (OxLDL) and remove free $CuSO_4$, OxLDL was concentrated by ultrafiltration through a 100-kDa molecular weight cutoff filter and washed four times with PBS. Before use in experiments, OxLDL was sterile filtered through 0.22 μm PVDF filters.

2.3.2. Preparation of acrolein-conjugated LDL

2 mg protein/mL of LDL in PBS was incubated with 2 μM acrolein at 37 ºC for 24 h under nitrogen atmosphere. In order to isolate acrolein-conjugated LDL (Acro-LDL) and remove free acrolein, Acro-LDL was concentrated by ultrafiltration through a 100-kDa molecular weight cutoff filter and washed four times with PBS. Before use in experiments, Acro-LDL was sterile filtered through 0.22 μm PVDF filters.

2.3.3. Preparation of *Mtb*-modified LDL

Mtb CDC1551 was cultured at 37 °C in Middlebrook 7H9 liquid media supplemented with 10% oleate-albumin-dextrose-NaCl-catalase (OADC) to OD~0.7. LDL was added to the culture and the culture was incubated at 37 °C for one week. The *Mtb*-modified LDL (*Mtb*-LDL) was isolated from the culture medium by centrifugation, sterile filtration of the supernatant, and *Mtb*-LDL was further concentrated by ultrafiltration through a 100-kDa molecular weight cutoff filter and washed four times with PBS. This work was performed by Dr. Xinxin Yang.

2.4. Determination of protein concentration

Protein concentrations were determined using the Bradford protein assay.[37] BSA was used as the standard in this assay.

2.5. Agarose gel electrophoresis of intact LDL and modified LDL

Agarose gel electrophoresis was performed in 0.06 M barbital buffer (pH 8.6) at 60 V, 4 ºC. Intact LDL and modified LDL were separated on a 0.8% agarose gel. A 0.8% agarose gel was made by melting agarose powder into 0.06 M barbital buffer, followed by pouring the solution into a casting tray and cooling at rt until the gel was completely solidified. After the samples were separated on the gel, the gel was stained with 0.28% (w/v) Sudan Red 7B in 95% MeOH/DI H_2O. The stain solution was stirred for 24 h and filtered before use. After staining the gel for 17 h, the gel was destained in 75% MeOH/DI H_2O until the bands were visible.

2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of intact LDL and modified LDL

SDS-PAGE was used to analyze apolipoprotein B (apoB) fragmentation. Intact LDL and modified LDL were separated on an SDS-PAGE gel. The stacking and running gels were 3.5% and 6% polyacrylamide, respectively. The molecular weights in the protein standard ranged from 30-460 kDa. The voltage at first was 50 V. When the lines were horizontal, the voltage was adjusted to 100 V. The gel was stained with Coomassie blue (0.1% Coomassie bue, 50% MeOH, 10% AcOH, and 40% DI H_2O) for 40 min and destained using destain I (50% MeOH, 10%

AcOH, and 40% DI H_2O) for 17 h, followed by destain II (5% MeOH, 7% AcOH, and 88% DI H_2O) to swell the gel.

2.7. Western blot analysis of intact LDL and modified LDL

Equal amounts of intact LDL and modified LDL (5 μg protein) were separated on 6% SDS-PAGE gels and electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes at 60 V, 4 ºC for 2 h. LDL commercial (Alfa Aesar, J65591) and OxLDL commercial (Alfa Aesar, J65039) were also used in this experiment. The primary antibodies were: anti-apoB antibody (H-300) (Santa Cruz, sc-25542), which is a rabbit polyclonal antibody raised against amino acids 1-300 of apoB of human origin; anti-LDL (copper oxidized) antibody (Abcam, ab14519), which is a rabbit polyclonal antibody and has strong reactivity with fully oxidized LDL, including copper oxidized LDL and malondialdehyde (MDA) oxidized LDL, but has weak reactivity with intact LDL; anti-LDL (MDA oxidized) antibody (Abcam, ab63976), which is a mouse monoclonal antibody that reacts with MDA oxidized LDL, but does not react with intact LDL and other human plasma proteins. The secondary antibodies were: goat anti-rabbit IgG (H+L)-horseradish peroxidase (HRP) conjugate (Biorad, 170-6515); goat anti-mouse IgG antibody, HRP conjugate (EMD Millipore, 12-349). PVDF membranes were treated with primary antibodies at 4 ºC for 17 h, and then treated with secondary antibodies at rt for 1 h. Finally, luminol/enhancer and peroxide buffer (immun-star HRP chemiluminescent substrate kit (Biorad, 170-5040)) were mixed in a 1:1 ratio and the membrane was incubated in the mixture for 3-5 min. The image was obtained by using a Typhoon 9400 scanner (Amersham Biosciences).

2.8. Tryptic digestion of apolipoprotein on PVDF membrane and MALDI-TOF analysis

Intact LDL or modified LDL (15 μg protein) was spotted on a PVDF membrane, washed twice with $CHCl_3$ by sonication for 10 min, and then air-dried. The membrane was soaked in 80 mM NH_4HCO_3 containing 20% CH_3CN and 10 mM dithiothreitol (DTT) at 56 ºC for 1 h. The reaction solution was removed, and the membrane was soaked in 80 mM NH_4HCO_3 containing 20% CH_3CN and 55 mM iodoacetamide (IAA) for 45 min in the dark. The reaction solution was removed, and the membrane was washed with DI H_2O. The membrane was incubated in 30 mM NH_4HCO_3 containing 70% CH_3CN and trypsin (5 μg). Peptides where then extracted twice with 1% trifluoroacetic acid (TFA)/70% CH_3CN and dried followed by resuspension with 0.1% formic acid/5% CH_3CN. A saturated solution of matrix α-cyano-4-hydroxycinnamic was prepared with 49.9% CH_3CN, 50% H_2O, and 0.1% formic acid. The samples were analyzed by MALDI-TOF mass spectra, obtained on a Bruker Autoflex II TOF/TOF instrument in positive ion mode. The obtained data were analyzed using Flex-Analysis and Sequence-Editor (Bruker Daltonics BioTools 3.0).

2.9. THP-1 macrophage formation

THP-1 cells, a human cell line from a one-year-old infant with acute monocytic leukemia, were maintained in RPMI-1640 medium with 10% FBS, 0.05 mM 2-mercaptoethanol, and 100 U/mL Pen Strep in an atmosphere of 5% CO_2/95% air. When the cell number reached 1×10^6 cells/mL, cells were plated in 6-well plates with a coverslip in each well, and were treated with 150 nM phorbol 12-myristate 13-acetate (PMA) in THP-1 growth media. After three days, the medium was removed, then the cells were rinsed twice with RPMI-1640 medium containing

10% FBS, and treated with fresh THP-1 growth media. After five days, the adherent cells could be used as differentiated macrophages.

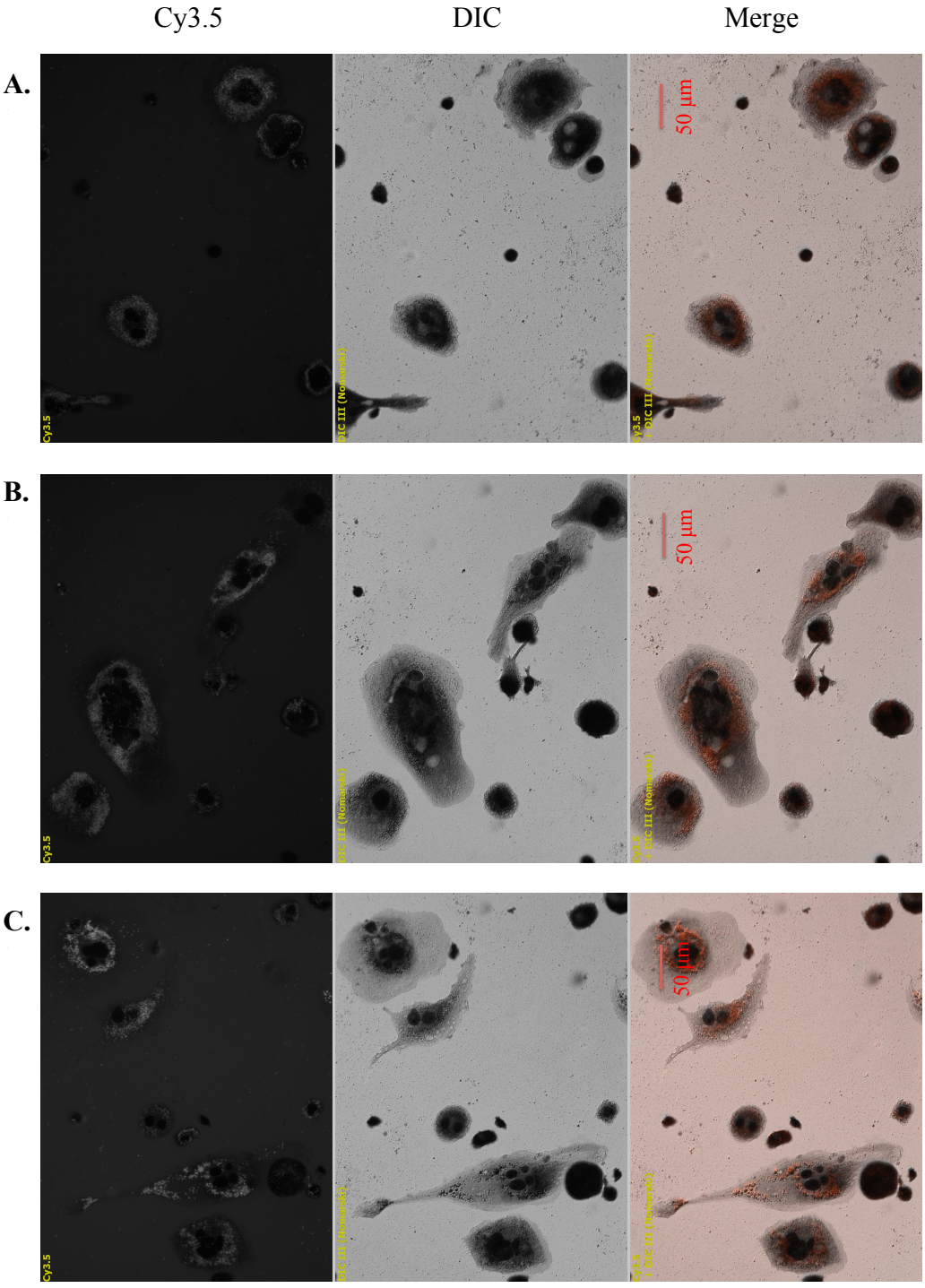
2.10. Lipid bodies

THP-1 macrophages were treated with intact LDL or modified LDL in THP-1 growth medium in 6-well plates for two days. 4% paraformaldehyde (PFA)-PBS solution was prepared by dissolving paraformaldehyde powder into PBS at 60 ºC and adjusting the pH to 6.9. Oil Red O stock solution was made by dissolving 300 mg Oil Red O powder in 100 mL isopropanol, and a working solution was made by mixing three parts of Oil Red O stock solution with two parts of DDI water. After removing the medium from the wells, cells were gently rinsed with sterile PBS twice, and were fixed in 4% PFA-PBS solution for 30 min. THP-1 macrophages were stained with freshly prepared Oil Red O working solution for 20 min at rt, and counterstained with hematoxylin for 3 min. Coverslips containing cells were rinsed with rt tap water until the water rinsed off clear, mounted on glass slides, and observed under an inverted microscope (Axiovert 200M Inverted Microscope, 20x objective, 10x eyepiece, 0.75 numerical aperture). Lipid bodies were stained red and nuclei were stained blue. Cell fluorescence was quantified using ImageJ software.

Chapter 3 Results and Discussion

3.1. The function of *Mtb*-modified LDL

LDL and modified LDL, the main carrier of plasma cholesterol, can cause macrophages to accumulate cholesterol and to become foamy macrophages. THP-1 macrophages were incubated with or without LDL, OxLDL, and Acro-LDL for 2 days after 3 days PMA culture and 5 days resting. Twenty continuous vision fields were selected and cell number as well as cell fluorescence were quantified. Figure 3-1 only showed one vision field among 10 continuous vision fields. Differential interference contrast (DIC) could convert gradients in specimen optical path length into amplitude differences, so that the contrast in unstained, transparent samples could be enhanced. The cell fluorescence in the Cy3.5 detection channel is indicated in red when merge. The results were in Table 3-1. The experiment was previously conducted twice, but the data was inconclusive. The data presented in Table 3-1 was from one representative experiment. We found that, after adding LDL, OxLDL, or Acro-LDL to macrophages, the average fluorescence per cell increased extremely, which demonstrated the number of lipid bodies did indeed increase after macrophages incubating with LDL, OxLDL, or Acro-LDL. However, not much difference of cell fluorescence was observed between intact LDL, OxLDL, and Acro-LDL. Then, the macrophages were incubated with *Mtb*-LDL at the same condition (performed by Dr. Xinxin Yang). We found that the cell fluorescence was much higher in macrophages incubated with *Mtb*-LDL than macrophages incubated with intact LDL, OxLDL, or Acro-LDL (data not shown). These results indicated that *Mtb*-LDL did indeed induce foamy macrophage formation, and compared with intact LDL, Acro-LDL, or OxLDL, *Mtb*-LDL could contribute much more to lipid bodies formation in macrophages.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DIC
Merge
A.
B.
C.
50 µm

D.

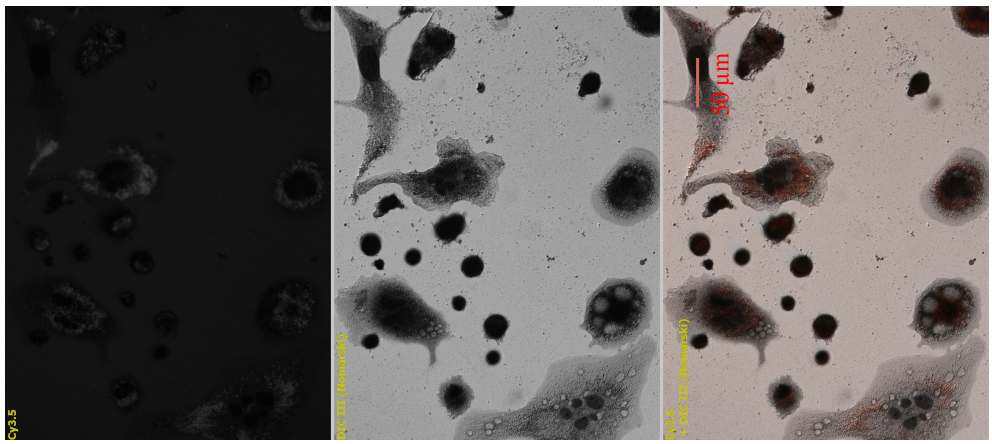


Figure 3-1. Inverted microscopy of THP-1 macrophages incubated with or without intact LDL, Acro-LDL, and OxLDL. (**A**) THP-1 macrophages without adding LDL. (**B**) THP-1 macrophages incubated with intact LDL. (**C**) THP-1 macrophages incubated with Acro-LDL. (**D**) THP-1 macrophages incubated with OxLDL.

	Control	Intact LDL	Acro-LDL	OxLDL
Total cell number	75	59	54	22
Average fluorescence per cell (a.u.)	52,958	535,880	728,029	1,033,748

Table 3-1. Cell fluorescence. The cells and cell fluorescence were counted and summed up from 10 continuous vision fields of the same experiment. This experiment was performed once.

3.2. The characterization of *Mtb*-modified LDL

3.2.1. LDL purification

LDL was isolated from culture supernatants of Hep G2 cells by sequential density ultracentrifugation. Hep G2 cells accumulated not only LDL but also high-density lipoprotein (HDL) in the culture medium. Also, bovine serum albumin (BSA), a major component of FBS, needed to be removed as well. Separation was accomplished by adjusting the density of the medium between centrifugations to allow sequential floatation of the individual lipoprotein fractions. Since the density of HDL was greater than that of LDL, we added potassium bromide so that the density of the solution was between the density of LDL and HDL. In this way, after ultracentrifugation, LDL was segregated into the top fraction, while HDL and albumin were in the bottom fraction.

Figure 3-2 showed SDS-PAGE gels of LDL. The molecular weight of apoB in LDL is about 500 kDa (the arrow pointed out the apoB band). In Figure 3-2A, the large and dark blue band was BSA. Comparing LDL in both gels, apoB band was the only obvious band in Fig 3-2B, whereas many extra bands appeared in Fig 3-2A. Therefore, LDL was purified after ultracentrifugation.

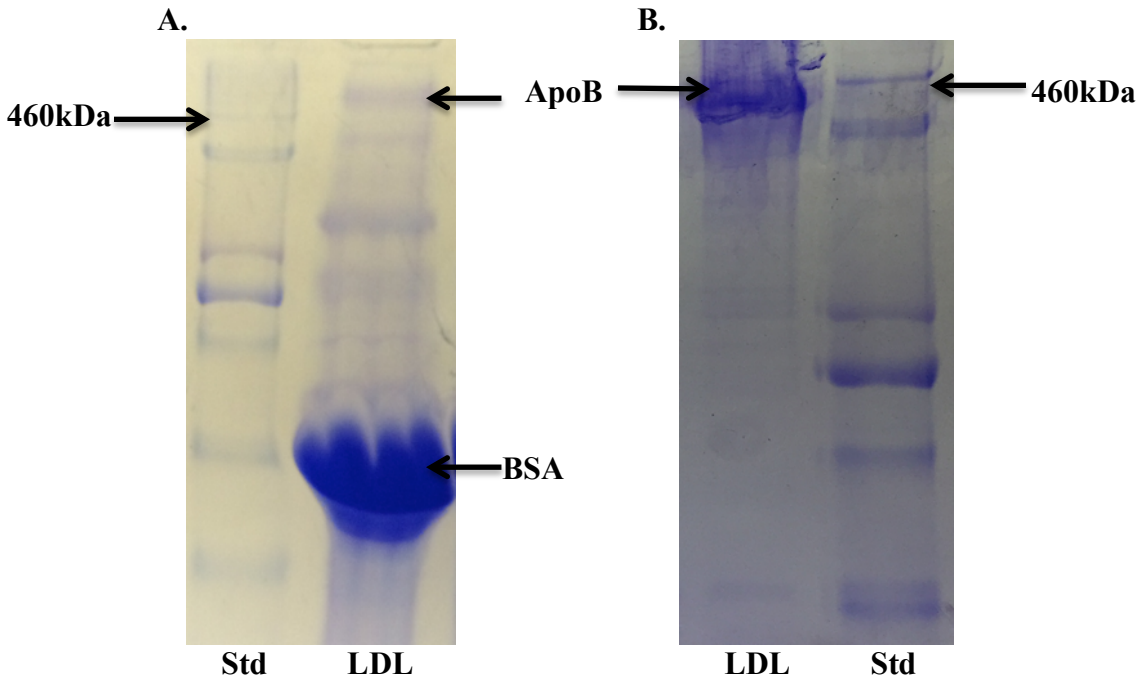


Figure 3-2. SDS-PAGE gels of LDL before and after ultracentrifugation. (**A**) Before ultracentrifugation, the sample contained a large amount of BSA and other proteins. (**B**) After ultracentrifugation, BSA and other proteins were removed and LDL was purified.

3.2.2. The difference of size or charge in *Mtb*-modified LDL and other forms of LDL

Relative electrophoretic mobility was determined by agarose gel electrophoresis. The mobility of the band in gel is influenced by both charge and size of the particles. The more negative charge or the smaller size the particle has, the closer the particle is able to move to the positively-charged anode. Compared with intact LDL, OxLDL, and Acro-LDL, the mobility of *Mtb*-LDL was decreased (Figure 3-3). This result suggested that, the charge of *Mtb*-LDL was more positive and the size of *Mtb*-LDL was larger. Therefore, modification of LDL in *Mtb* cells was different from oxidation or acrolein conjugation.

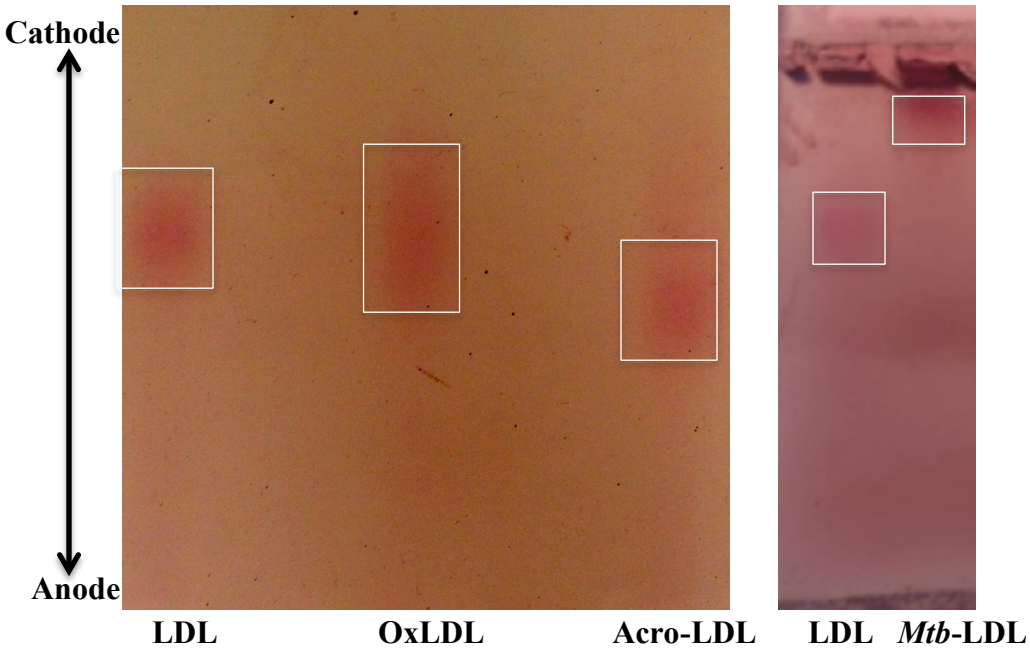
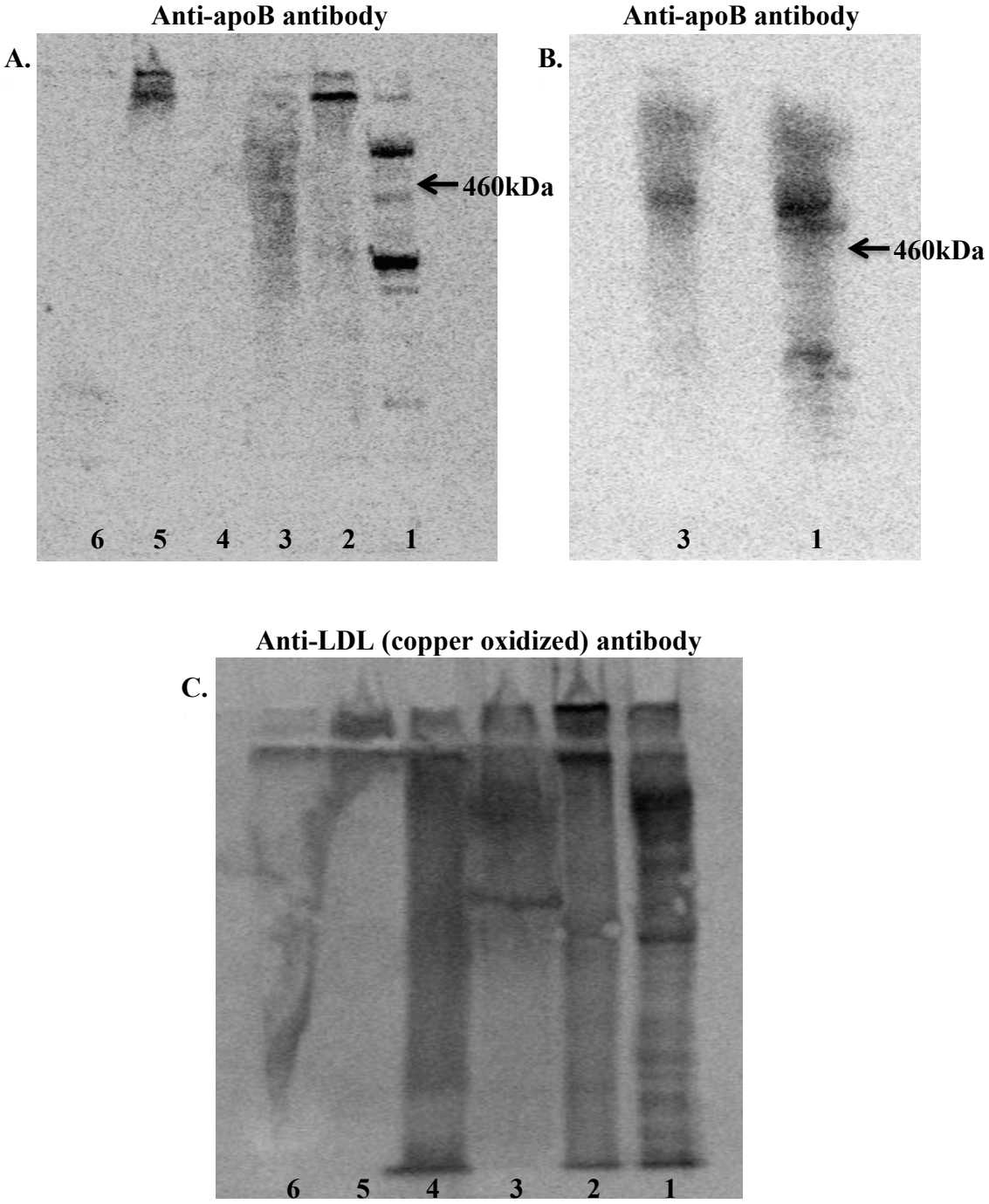


Figure 3-3. Agarose gels of intact LDL and modified LDL. The gel was stained with Sudan Red 7B. Each lane was loaded with 1 mg protein of LDL or modified LDL with 95% BSA.

3.2.3. The difference of apoB in *Mtb*-modified LDL and other forms of LDL

Identity of apoB in *Mtb*-LDL was examined by Western blot. Molecular weight 460 kDa was marked based on the overlay of the pre-stained protein standard. The samples we used were LDL commercial, LDL commercial, OxLDL commercial, LDL (after ultracentrifugation), OxLDL synthesized, Acro-LDL synthesized, and *Mtb*-LDL. All of the samples were BSA free except for *Mtb*-LDL (Figure 3-5). The percentage of BSA in *Mtb*-LDL was about 70% based on the bands in SDS gel (Figure 3-5). In Figure 3-4A, as expected, apoB in intact LDL could be detected by anti-apoB antibody. The reason why apoB in LDL commercial showed not only molecular weight 500 kDa but also several other molecular weights was that some apoB was

degraded during storage. Also, due to long time storage, in Figure 3-4A, no clear band with molecular weight 500 kDa was observed in ApoB of LDL we purified, but in Figure 3-4B, apoB in LDL we purified (fresh) showed an apparent molecular weight 500 kDa. OxLDL commercial and Acro-LDL could also be detected by anti-apoB antibody, and both molecular weights were more than 1000 kDa, which means apoB in OxLDL and AcroLDL was cross-linked or aggregated (Figure 3-4A). ApoB in *Mtb*-LDL was not detected by anti-apoB antibody in this gel (Figure 3-4A). But from Dr. Xinxin Yang's Western blot analysis, apoB in *Mtb*-LDL could be detected by anti-apoB antibody, and the experiment was repeated for multiple times. The samples we used were from the same batch, but the experiments were conducted weeks apart. So the possible reason why apoB in *Mtb*-LDL was not detected by anti-apoB antibody in this gel could be protein degradation. In Figure 3-4C, anti-LDL (copper oxidized) antibody could recognize apoB in intact LDL, OxLDL and Acro-LDL, but it could not recognize or could recognize a very small amount of apoB in *Mtb*-LDL, which suggested that modification in *Mtb* cells is different from oxidation. In Figure 3-4D, anti-LDL (MDA oxidized) antibody could only recognize apoB in Acro-LDL. This result suggested that modification in *Mtb* cells is not the same as acrolein conjugation. Also, because anti-LDL (MDA oxidized) antibody was monoclonal, whereas anti-apoB antibody and anti-LDL (copper oxidized) antibody were polyclonal, the band in Figure 3-4C was clearer than that in other figures. To sum up, in Western blot analysis, we demonstrated that *Mtb*-LDL was different from OxLDL and Acro-LDL.


Anti-apoB antibody
A.
460kDa
6 5 4 3 2 1
Anti-apoB antibody
B.
460kDa
3 1
Anti-LDL (copper oxidized) antibody
C.
6 5 4 3 2 1

Anti-LDL (MDA oxidized) antibody

D.

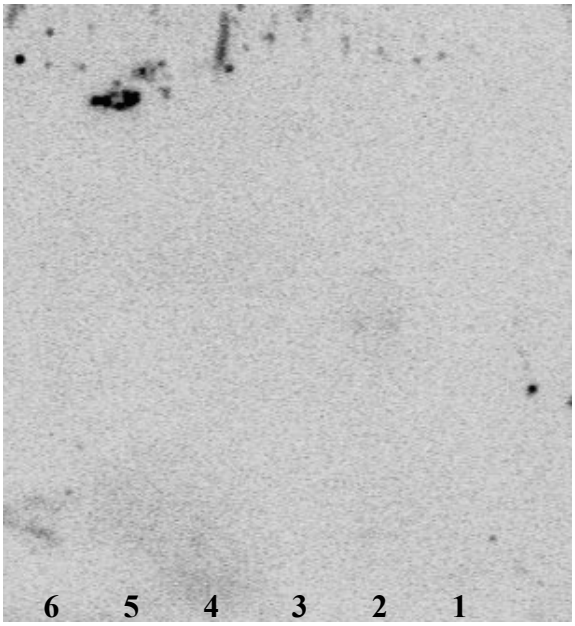


Figure 3-4. Western blot analysis of apoB in intact LDL and modified LDL. In Figure A, B, C, D, each number represents a different lane with different sample. 1: LDL commercial 2: OxLDL commercial 3: LDL (after ultracentrifugation) 4: OxLDL synthesized 5: Acro-LDL synthesized 6: *Mtb*-LDL. The samples in Figure B were fresh, but the samples in Figure A, C, D were 2-3 months old. Each lane was loaded with 5 μg protein of LDL or modified LDL.

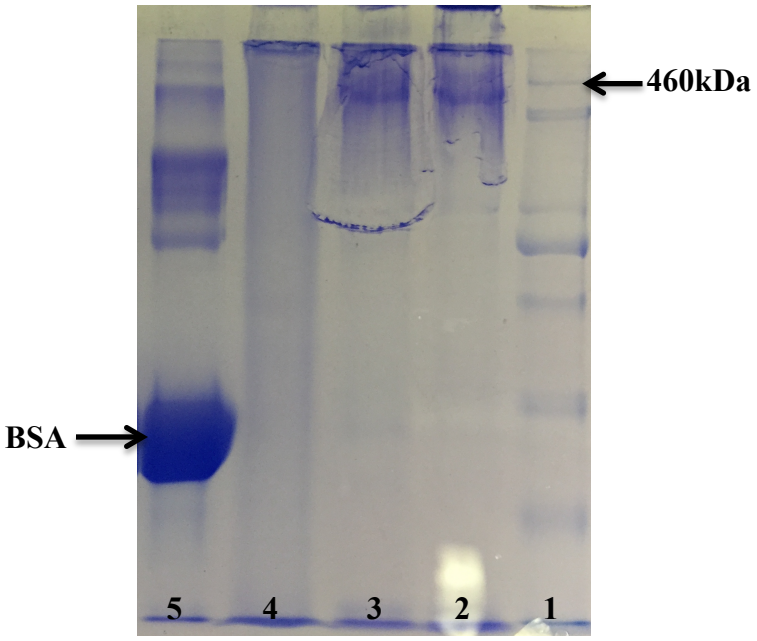


Figure 3-5. SDS-PAGE of apoB in intact LDL and modified LDL. Each number represents a different lane with different sample. 1: Protein standard 2: LDL (after ultracentrifugation) 3: Acro-LDL synthesized 4: OxLDL synthesized 5: *Mtb*-LDL

3.2.4. Analysis of apoB in *Mtb*-modified LDL by tryptic digestion on PVDF membrane and MALDI-TOF

To search for modification of amino acid residues of apoB100, the main protein component, in *Mtb*-LDL, an on-membrane sample preparation procedure was established. At first, a conventional proteome technique, in-gel tryptic digestion, was used to analyze apoB100. However, the sequence coverage was very low (<10%) (data not shown here). Then an alternative method, on-membrane tryptic digestion, was employed in this experiment.

It has been demonstrated that PVDF membrane has some advantages for processing lipoprotein.[38] PVDF membrane could adsorb lipids and proteins, but lipids with low molecular weight could be washed out by $CHCl_3$, which reduces the interference from lipids. Also, PVDF membrane is available for enzymic digestion in the presence of 80% CH_3CN. This method greatly improved the sequence coverage in MALDI-TOF analysis because of increased tryptic digestion efficiency and peptide recovery. In Table 3-2, the samples we used were LDL commercial and *Mtb*-LDL, among which LDL commercial was fresh without BSA while *Mtb*-LDL was 2 months old with BSA in it. Figure 3-6 and 3-7 showed the matched sequences of apoB100 in intact LDL (commercial) and *Mtb*-LDL.

Several studies have shown that some amino acid residues of apoB100 were modified in OxLDL and Acro-LDL.[36] Some residues were converted to their oxidized forms by the addition of an oxygen atom (+ 16 Da). 4-hydroxynonenal (HNE) and acrolein are lipid peroxidation products. When HNE was bound to proteins, a 156 Da shift was observed for all of the peptides containing the modified residues. Acrolein could react with Lys to form FDP-Lys (+ 94 Da) and MP-Lys (+ 76 Da). For example, in copper oxidized LDL, certain His residues were detected with a 16 Da or 156 Da shift in the MS/MS spectrum, certain Trp residues had a 16 Da mass shift, certain Lys residues had a 76 Da mass shift; in Acro-LDL, certain Lys residues had a 76 Da or 94 Da mass shift. In our study, in order to find modified amino acid residues in apoB100 of *Mtb*-LDL, we first added a mass to certain residues of apoB and digested it using Sequence Editor software, and then matched with experimentally digested peptides. Table 3-3 showed the matched peptides in intact LDL (commercial) or *Mtb*-LDL with theoretically digested intact apoB or modified apoB. The peaks which are the same in MALDI-TOF spectra of intact LDL

(commercial) and *Mtb*-LDL were indicated in red. Figure 3-8 and 3-9 were the MALDI-TOF spectrum of intact LDL (commercial) and *Mtb*-LDL, respectively. There were 69 potential modified peptides with a 76 Da mass shift on Lys residues; 75 potential modified peptides with a 94 Da mass shift on Lys residues; 11 potential modified peptides with a 16 Da mass shift on His residues; 5 potential modified peptides with a 16 Da mass shift on Trp residues; 21 potential modified peptides with a 156 Da mass shift on His residues; and 4 potential modified peptides with a 156 Da mass shift on Cys residues. For some peaks, there are several matched potential modified peptides, which indicated that those peaks have higher possibility to be modified.

Several peaks with high intensity in MALDI-TOF spectrum of *Mtb*-LDL were analyzed by MALDI-TOF/TOF. In Figure 3-10 A, B, C, and D, due to no or just few fragment ion, the peaks with m/z 1001, 1481, 1640, and 1882 could not be identified as peptides. They could be the lipids in *Mtb*. In Figure 3-9 E and F, even if there were several fragment ions, no *de novo* sequencing result was found by the software. The reason could be the incomplete fragment ions. In the future, LC-MS/MS with high resolution could be used to identify the peaks in *Mtb*-LDL MALDI-TOF spectrum.

	Intact LDL (commercial)	*Mtb*-LDL
Sequence coverage	40.4%	21.4%

Table 3-2. Sequence coverage in MALDI-TOF analysis of LDL.

```
1    mdpprpalla llalpallll llagaraeee mlenvslvcp kdatrfkhlr kytynyeaes
61   ssgvpgtads rsatrinckv elevpqlcsf ilktsqctlk evygfnpegk allkktknse
121  efaaamsrye lklaipegkq vflypekdep tyilnikrgi isallvppet eeakqvlfld
181  tvygncsthf tvktrkgnva teisterdlg qcdrfkpirt gisplalikg mtrplstlis
241  ssqscqytld akrkhvaeai ckeqhlflpf sykknkygmva qvtqtlkled tpkinsrffg
301  egtkkmglaf estkstsppk qaeavlktlq elkkltiseq niqranlfnk lvtelrglsd
361  eavtsllpql ievsspitlq alvqcgqpqc sthilqwlkr vhanpllidv vtylvalipe
421  psaqqlreif nmardqrsra tlyalshavn nyhktnptgt qelldianyl meqiqddctg
481  dedytylilr vignmgqtme qltpelkssi lkcvqstkps lmiqkaaiqa lrkmepkdkd
541  qevllqtfld daspgdkrla aylmlmrsps qadinkivqi lpweqneqvk nfvashiani
601  lnseeldiqd lkklvkealk esqlptvmdf rkfsrnyqly ksvslpsldp asakiegnli
661  fdpnnylpke smlkttltaf gfasadliei glegkgfept lealfgkqgf fpdsvnkaly
721  wvngqvpdgv skvlvdhfgy tkddkheqdm vngimlsvek likdlkskev pearaylril
781  geelgfaslh dlqllgklll mgartlqgip qmigevirkg skndfflhyi fmenafelpt
841  gaglqlqiss sgviapgaka gvklevanmq aelvakpsvs vefvtnmgii ipdfarsgvq
901  mntnffhesg leahvalkag klkfiipspk rpvkllsggn tlhlvsttkt evipplienr
961  qswsvckqvf pglnyctsga ysnasstdsa syypltgdtr lelelrptge ieqysvsaty
1021 elqredralv dtlkfvtqae gakqteatmt fkynrqsmtl ssevqipdfd vdlgtilrvn
1081 destegktsy rltldiqnkk itevalmghl scdtkeerki kgvisiprlq aearseilah
1141 wspaklllqm dssataygst vskrvawhyd eekiefewnt gtnvdtkkmt snfpvdlsdy
1201 pkslhmyanr lldhrvpqtd mtfrhvgskl ivamsswlqk asgslpytqt lqdhlnslke
1261 fnlqnmglpd fhipenlflk sdgrvkytln knslkieipl pfggkssrdl kmletvrtpa
1321 lhfksvgfhl psrefqvptf tipklyqlqv pllgvldlst nvysnlynws asysggntst
1381 dhfslraryh mkadsvvdll synvqgsget tydhkntftl scdgslrhkf ldsnikfshv
1441 eklgnnpvsk gllifdasss wgpqmsasvh ldskkkqhlf vkevkidgqf rvssfyakgt
1501 yglscqrdpn tgrlngesnl rfnssylqgt nqitgryedg tlsltstsdl qsgiikntas
1561 lkyenyeltl ksdtngkykn fatsnkmdmt fskqnallrs eyqadyeslr ffsllsgsln
1621 shglelnadi lgtdkinsga hkatlrigqd gistsattnl kcsllvlene lnaelglsga
1681 smklttngrf rehnakfsld gkaaltelsl gsayqamilg vdsknifnfk vsqeglklsn
1741 dmmgsyaemk fdhtnslnia glsldfsskl dniyssdkfy kqtvnlqlqp yslvttlnsd
1801 lkynaldltn ngklrleplk lhvagnlkga yqnneikhiy aissaalsas ykadtvakvq
1861 gvefshrlnt diaglasaid mstnynsdsl hfsnvfrsvm apftmtidah tngngklalw
1921 gehtgqlysk fllkaeplaf tfshdykgst shhlvsrksi saalehkvsa lltpaeqtgt
1981 wklktqfnnn eysqdldayn tkdkigvelt grtladltll dspikvplll sepiniidal
2041 emrdavekpq eftivafvky dknqdvhsin lpffetlqey fernrqtiiv vlenvqrnlk
2101 hinidqfvrk yraalgklpq qandylnsfn werqvshake kltaltkkyr itendiqial
2161 ddakinfnek lsqlqtymiq fdqyikdsyd lhdlkiaian iideiieklk sldehyhirv
2221 nlvktihdlh lfienidfnk sgsstaswiq nvdtkyqiri qiqeklqqlk rhiqnidiqh
2281 lagklkqhie aidvrvlldq lgttisferi ndvlehvkhf vinligdfev aekinafrak
2341 vhelieryev dqqiqvlmdk lvelahqykl ketiqklsnv lqqvkikdyf eklvgfidda
2401 vkklnelsfk tfiedvnkfl dmlikklksf dyhqfvdetn dkirevtqrl ngeiqalelp
2461 qkaealklfl eetkatvavy leslqdtkit liinwlqeal ssaslahmka kfretledtr
2521 drmyqmdiqq elqrylslvg qvystlvtyi sdwwtlaakn ltdfaeqysi qdwakrmkal
2581 veqgftvpei ktilgtmpaf evslqalqka tfqtpdfivp ltdlripsvq infkdlknik
2641 ipsrfstpef tilntfhips ftidfvemkv kiirtidqml nselqwpvpd iylrdlkved
2701 iplaritlpd frlpeiaipe fiiptlnlnd fqvpdlhipe fqlphishti evptfgklys
2761 ilkiqsplft ldanadigng ttsaneagia asitakgesk levlnfdfqa naqlsnpkin
2821 plalkesvkf sskylrtehg semlffgnai egksntvasl htekntlels ngvivkinnq
2881 ltldsntkyf hklnipkldf ssqadlrnei ktllkaghia wtssgkgswk wacprfsdeg
2941 thesqisfti egpltsfgls nkinskhlrv nqnlvyesgs lnfskleiqs qvdsqhvghs
3001 vltakgmalf gegkaeftgr hdahlngkvi gtlknslffs aqpfeitast nnegnlkvrf
3061 plrltgkidf lnnyalflsp saqqaswqvs arfnqykynq nfsagnneni meahvginge
3121 anldflnipl tipemrlpyt iittpplkdf slwektglke flkttkqsfd lsvkaqykkn
```

3181 khrhsitnpl avlcefisqs iksfdrhfek nrnnaldfvt ksynetkikf dkykaekshd
3241 elprtfqipg ytvpvvnvev spftiemsaf gyvfpkavsm psfsilgsdv rvpsytlilp
3301 slelpvlhvp rnlklslpdf kelctishif ipamgnityd fsfkssvitl ntnaelfnqs
3361 divahllsss ssvidalqyk legttrltrk rglklatals lsnkfvegsh nstvslttkn
3421 mevsvatttk aqipilrmnf kqelngntks kptvsssmef kydfnssmly stakgavdhk
3481 lslesltsyf siesstkgdv kgsvlsreys gtiaseanty lnskstrssv klqgtskidd
3541 iwnlevkenf ageatlqriy slwehstknh lqleglfftn gehtskatle lspwqmsalv
3601 qvhasqpssf hdfpdlgqev alnantknqk irwknevrih sgsfqsqvel sndqekahld
3661 iagsleghlr flkniilpvy dkslwdflkl dvttsigrrq hlrvstafvy tknpngysfs
3721 ipvkvladkf iipglklndl nsvlvmptfh vpftdlqvps ckldfreiqi ykklrtssfa
3781 lnlptlpevk fpevdvltky sqpedslipf feitvpesql tvsqftlpks vsdgiaaldl
3841 navankiadf elptiivpeq tieipsikfs vpagivipsf qaltarfevd spvynatwsa
3901 slknkadyve tvldstcsst vqfleyelnv lgthkiedgt lasktkgtfa hrdfsaeyee
3961 dgkyeglqew egkahlniks paftdlhlry qkdkkgists aaspavgtvg mdmdedddfs
4021 kwnfyyspqs spdkkltifk telrvresde etqikvnwee eaasglltsl kdnvpkatgv
4081 lydyvnkyhw ehtgltlrev ssklrrnlqn naewvyqgai rqiddidvrf qkaasgttgt
4141 yqewkdkaqn lyqelltqeg qasfqglkdn vfdglvrvtq efhmkvkhli dslidflnfp
4201 rfqfpgkpgi ytreelctmf irevgtvlsq vyskvhngse ilfsyfqdlv itlpfelrkh
4261 klidvismyr ellkdlskea qevfkaiqsl kttevlrnlq dllqfifqli ednikqlkem
4321 kftylinyiq deintifsdy ipyvfkllke nlclnlhkfn efiqnelqea sqelqqihqy
4381 imalreeyfd psivgwtvky yeleekivsl iknllvalkd fhseyivsas nftsqlssqv
4441 eqflhrniqe ylsiltdpdg kgkekiaels ataqeiiksq aiatkkiisd yhqqfryklq
4501 dfsdqlsdyy ekfiaeskrl idlsiqnyht fliyitellk klqsttvmnp ymklapgelt
4561 iil

Figure 3-6. Distribution of tryptic digested peptides in intact LDL (commercial) identified by MALDI-TOF analysis. Identified peptides are indicated in red.

```
1    mdpprpalla llalpallll llagaraeee mlenvslvcp kdatrfkhlr kytynyeaes
61   ssgvpgtads rsatrinckv elevpqlcsf ilktsqctlk evygfnpegk allkktknse
121  efaaamsrye lklaipegkq vflypekdep tyilnikrgi isallvppet eeakqvlfld
181  tvygncsthf tvktrkgnva teisterdlg qcdrfkpirt gisplalikg mtrplstlis
241  ssqscqytld akrkhvaeai ckeqhlflpf syknkygmva qvtqtlkled tpkinsrffg
301  egtkkmglaf estkstsppk qaeavlktlq elkkltiseq niqranlfnk lvtelrglsd
361  eavtsllpql ievsspitlq alvqcgqpqc sthilqwlkr vhanpllidv vtylvalipe
421  psaqqlreif nmardqrsra tlyalshavn nyhktnptgt qelldianyl meqiqddctg
481  dedytylilr vignmgqtme qltpelkssi lkcvqstkps lmiqkaaiqa lrkmepkdkd
541  qevllqtfld daspgdkrla aylmlmrsps qadinkivqi lpweqneqvk nfvashiani
601  lnseeldiqd lkklvkealk esqlptvmdf rkfsrnyqly ksvslpsldp asakiegnli
661  fdpnnylpke smlkttltaf gfasadliei glegkgfept lealfgkqgf fpdsvnkaly
721  wvngqvpdgv skvlvdhfgy tkddkheqdm vngimlsvek likdlkskev pearaylril
781  geelgfaslh dlqllgklll mgartlqgip qmigevirkg skndfflhyi fmenafelpt
841  gaglqlqiss sgviapgaka gvklevanmq aelvakpsvs vefvtnmgii ipdfarsgvq
901  mntnffhesg leahvalkag klkfiipspk rpvkllsggn tlhlvsttkt evipplienr
961  qswsvckqvf pglnyctsga ysnasstdsa syypltgdtr lelelrptge ieqysvsaty
1021 elqredralv dtlkfvtqae gakqteatmt fkynrqsmtl ssevqipdfd vdlgtilrvn
1081 destegktsy rltldiqnkk itevalmghl scdtkeerki kgvisiprlq aearseilah
1141 wspaklllqm dssataygst vskrvawhyd eekiefewnt gtnvdtkkmt snfpvdlsdy
1201 pkslhmyanr lldhrvpqtd mtfrhvgskl ivamsswlqk asgslpytqt lqdhlnslke
1261 fnlqnmglpd fhipenlflk sdgrvkytln knslkieipl pfggkssrdl kmletvrtpa
1321 lhfksvgfhl psrefqvptf tipklyqlqv pllgvldlst nvysnlynws asysggntst
1381 dhfslraryh mkadsvvdll synvqgsget tydhkntftl scdgslrhkf ldsnikfshv
1441 eklgnnpvsk gllifdasss wgpqmsasvh ldskkkqhlf vkevkidgqf rvssfyakgt
1501 yglscqrdpn tgrlngesnl rfnssylqgt nqitgryedg tlsltstsdl qsgiikntas
1561 lkyenyeltl ksdtngkykn fatsnkmdmt fskqnallrs eyqadyeslr ffsllsgsln
1621 shglelnadi lgtdkinsga hkatlrigqd gistsattnl kcsllvlene lnaelglsga
1681 smklttngrf rehnakfsld gkaaltelsl gsayqamilg vdsknifnfk vsqeglklsn
1741 dmmgsyaemk fdhtnslnia glsldfsskl dniyssdkfy kqtvnlqlqp yslvttlnsd
1801 lkynaldltn ngklrleplk lhvagnlkga yqnneikhiy aissaalsas ykadtvakvq
1861 gvefshrlnt diaglasaid mstnynsdsl hfsnvfrsvm apftmtidah tngngklalw
1921 gehtgqlysk fllkaeplaf tfshdykgst shhlvsrksi saalehkvsa lltpaeqtgt
1981 wklktqfnnn eysqdldayn tkdkigvelt grtladltll dspikvplll sepiniidal
2041 emrdavekpq eftivafvky dknqdvhsin lpffetlqey fernrqtiiv vlenvqrnlk
2101 hinidqfvrk yraalgklpq qandylnsfn werqvshake kltaltkkyr itendiqial
2161 ddakinfnek lsqlqtymiq fdqyikdsyd lhdlkiaian iideiieklk sldehyhirv
2221 nlvktihdlh lfienidfnk sgsstaswiq nvdtkyqiri qiqeklqqlk rhiqnidiqh
2281 lagklkqhie aidvrvlldq lgttisferi ndvlehvkhf vinligdfev aekinafrak
2341 vhelieryev dqqiqvlmdk lvelahqykl ketiqklsnv lqqvkikdyf eklvgfidda
2401 vkklnelsfk tfiedvnkfl dmlikklksf dyhqfvdetn dkirevtqrl ngeiqalelp
2461 qkaealklfl eetkatvavy leslqdtkit liinwlqeal ssaslahmka kfretledtr
2521 drmyqmdiqq elqrylslvg qvystlvtyi sdwwtlaakn ltdfaeqysi qdwakrmkal
2581 veqgftvpei ktilgtmpaf evslqalqka tfqtpdfivp ltdlripsvq infkdlknik
2641 ipsrfstpef tilntfhips ftidfvemkv kiirtidqml nselqwpvpd iylrdlkved
2701 iplaritlpd frlpeiaipe fiiptlnlnd fqvpdlhipe fqlphishti evptfgklys
2761 ilkiqsplft ldanadigng ttsaneagia asitakgesk levlnfdfqa naqlsnpkin
2821 plalkesvkf sskylrtehg semlffgnai egksntvasl htekntlels ngvivkinnq
2881 ltldsntkyf hklnipkldf ssqadlrnei ktllkaghia wtssgkgswk wacprfsdeg
2941 thesqisfti egpltsfgls nkinskhlrv nqnlvyesgs lnfskleiqs qvdsqhvghs
3001 vltakgmalf gegkaeftgr hdahlngkvi gtlknslffs aqpfeitast nnegnlkvrf
3061 plrltgkidf lnnyalflsp saqqaswqvs arfnqykynq nfsagnneni meahvginge
3121 anldflnipl tipemrlpyt iittpplkdf slwektglke flkttkqsfd lsvkaqykkn
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3181 khrhsitnpl avlcefisqs iksfdrhfek nrnnaldfvt ksynetkikf dkykaekshd
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3301 slelpvlhvp rnlklslpdf kelctishif ipamgnityd fsfkssvitl ntnaelfnqs
3361 divahllsss ssvidalqyk legttrltrk rglklatals lsnkfvegsh nstvslttkn
3421 mevsvatttk aqipilrmnf kqelngntks kptvsssmef kydfnssmly stakgavdhk
3481 lslesltsyf siesstkgdv kgsvlsreys gtiaseanty lnskstrssv klqgtskidd
3541 iwnlevkenf ageatlqriy slwehstknh lqleglfftn gehtskatle lspwqmsalv
3601 qvhasqpssf hdfpdlgqev alnantknqk irwknevrih sgsfqsqvel sndqekahld
3661 iagsleghlr flkniilpvy dkslwdflkl dvttsigrrq hlrvstafvy tknpngysfs
3721 ipvkvladkf iipglklndl nsvlvmptfh vpftdlqvps ckldfreiqi ykklrtssfa
3781 lnlptlpevk fpevdvltky sqpedslipf feitvpesql tvsqftlpks vsdgiaaldl
3841 navankiadf elptiivpeq tieipsikfs vpagivipsf qaltarfevd spvynatwsa
3901 slknkadyve tvldstcsst vqfleyelnv lgthkiedgt lasktkgtfa hrdfsaeyee
3961 dgkyeglqew egkahlniks paftdlhlry qkdkkgists aaspavgtvg mdmdedddfs
4021 kwnfyyspqs spdkkltifk telrvresde etqikvnwee eaasglltsl kdnvpkatgv
4081 lydyvnkyhw ehtgltlrev ssklrrnlqn naewvyqgai rqiddidvrf qkaasgttgt
4141 yqewkdkaqn lyqelltqeg qasfqglkdn vfdglvrvtq efhmkvkhli dslidflnfp
4201 rfqfpgkpgi ytreelctmf irevgtvlsq vyskvhngse ilfsyfqdlv itlpfelrkh
4261 klidvismyr ellkdlskea qevfkaiqsl kttevlrnlq dllqfifqli ednikqlkem
4321 kftylinyiq deintifsdy ipyvfkllke nlclnlhkfn efiqnelqea sqelqqihqy
4381 imalreeyfd psivgwtvky yeleekivsl iknllvalkd fhseyivsas nftsqlssqv
4441 eqflhrniqe ylsiltdpdg kgkekiaels ataqeiiksq aiatkkiisd yhqqfryklq
4501 dfsdqlsdyy ekfiaeskrl idlsiqnyht fliyitellk klqsttvmnp ymklapgelt
4561 iil

Figure 3-7. Distribution of tryptic digested peptides in *Mtb*-LDL identified by MALDI-TOF analysis. Identified peptides are indicated in red.

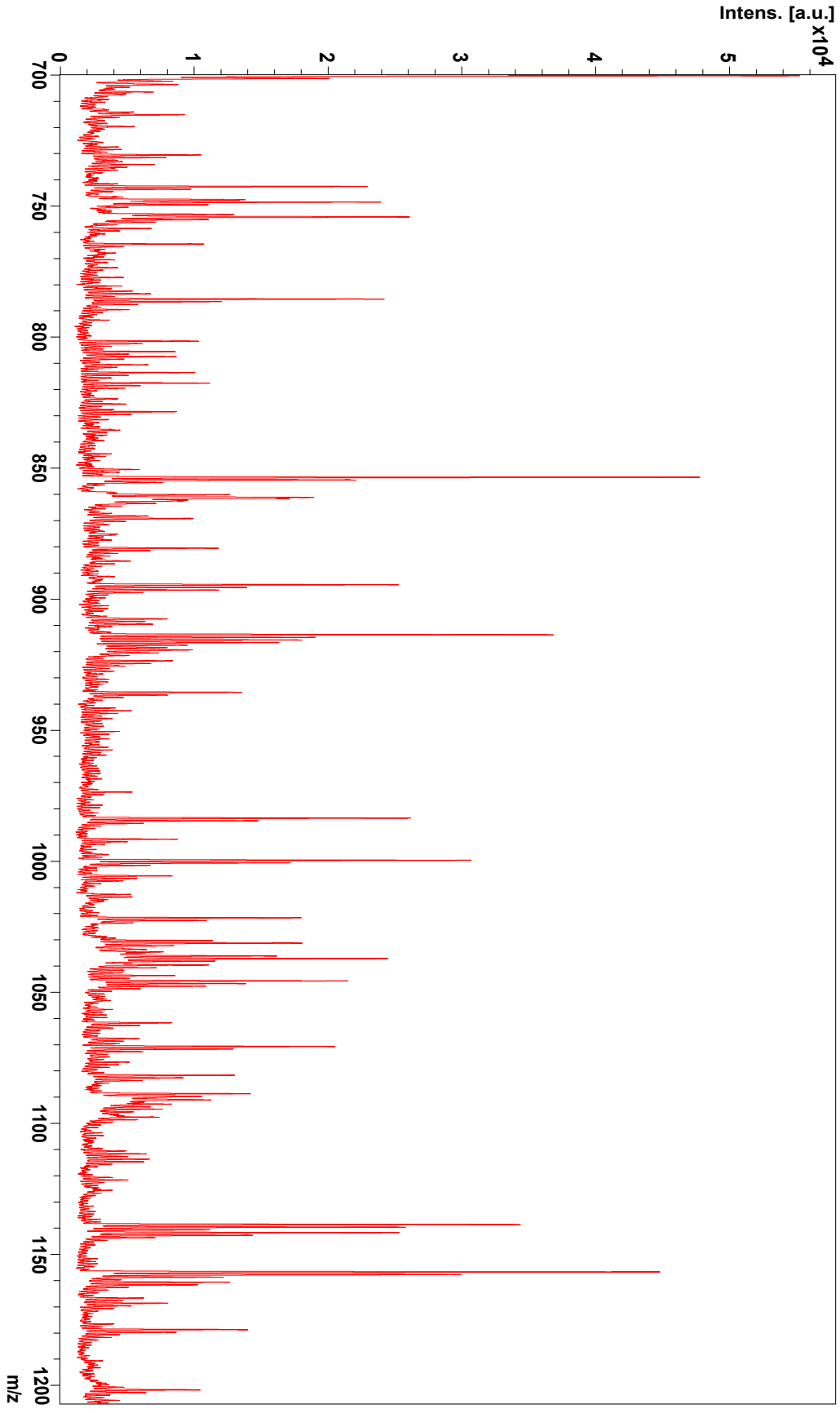
Peak #	m/z (*Mtb*-LDL)	Intensity (*Mtb*-LDL)	Matched Sequence	m/z (LDL)	Intensity (LDL)	Matched Sequence
1	700.2	133575.3		700.2	49012.2	
2				707.2	1424.0	
3	711.6	7064.4	AH(+16)LNIK			
4	712.5	22876.6	ETIQK(+94)			
5	714.2	9977.5	QNALLR/YTLNK(+76)	714.3	2436.0	QNALLR
6	715.2	22408.6	SDTNGK(+94)	715.2	5067.1	
7	719.8	7867.0		719.6	2174.7	
8	721.5	7205.9				
9	727.7	6324.1		727.5	1317.1	LAIPEGK
10				728.4	1284.8	
11				730.5	8999.1	LVTELR
12				731.4	1947.2	TLQELK
13	733.6	41265.5		733.1	1736.1	
14				734.1	3843.9	
15				736.3	1162.5	LYSILK
16				741.5	1543.4	SYNETK/GVISIPR
17				742.5	20393.0	AAIQALR
18	746.9	2764.8		746.6	1743.1	FSHVEK
19	747.9	23218.2		747.6	12031.7	
20				748.5	14882.7	MLETVR
21	753.1	30320.8		753.2	10229.6	
22	754.1	55354.9	EH(+156)NAK	754.2	19166.4	
23				756.2	2208.9	
24				758.5	3949.5	IQIQEK
25				764.5	8285.8	INFNEK/STRSSVK
26				770.5	1648.2	NLLVALK
27	771.7	5130.0				
28				773.5	1419.0	TELRVR/LLLMGAR
29	774.5	2638.3	LTALTKK			
30				777.2	1964.4	
31	780.6	10405.4	TSQCTLK	780.5	2015.5	TSQCTLK
32				782.5	2721.2	NIFNFK
33				783.5	2787.8	
34				785.5	22282.4	FFGEGTK/LQQLKR
35				789.5	2307.8	
36				801.5	7973.4	VSSFYAK/FIIPSPK/ALLKKTK
37	805.6	5478.3	ARYHMK	805.5	5552.6	ARYHMK
38	806.8	2217.1				
39				807.5	5186.9	
40				810.6	3361.6	AQIPILR
41				813.5	7614.6	TPALHFK
42	815.8	4537.7				
43	817.7	33159.4		817.5	8964.9	
44	818.5	7207.8	EVSSKLR			
45				823.5	1362.2	
46				825.5	1797.4	
47				827.6	1196.0	NIKIPSR
48				828.5	5842.9	NYQLYK/LGNNPVSK/FKHLRK
49				831.5	967.3	WKNEVR
50	834.3	1324.2	IQIQEK(+76)/K(+76)NK(+76)HR			
51	835.3	3352.2	SYNETK(+94)/ALVDTLK(+76)	835.4	1638.3	
52	837.6	1361.1	QSWSVCK			
53	839.7	5131.5				
54	840.6	10032.2	FSHVEK(+94)/			
55			INFNEK(+76)/STRSSVK(+76)			
56	841.2	2507.0				
57				844.5	1087.3	IGVELTGR
58	847.7	31425.4	W(+16)KNEVR/QHLFVK(+76)			
59				850.5	3164.0	EAQEVFK/LNELSFK/FIAESKR
60	851.5	1839.4	LHVAGNLK/AH(+156)LNIK			
61	853.4	2565.6	SHDELPR/ALVDTLK(+94)	853.5	44436.1	SHDELPR
62	860.2	25455.9		860.2	8304.6	
63	861.3	27943.6	ITLPDFR/FFGEGTK(+76)	861.1	8113.5	
64	864.6	1376.2	NLLVALK(+94)			
65	867.4	1873.7	INSKHLR			
66	868.3	4303.3	LRLEPLK	868.3	3678.2	
67	869.7	31883.2	SH(+16)DELPR/YQK(+94)DK(+94)/EIQIYK(+76)	869.3	5055.6	
68	874.3	2228.7	TSQCTLK(+94)			
69	875.2	3775.4	KMEPKDK/NFATSNK(+94)	875.3	1330.8	KMEPKDK
70				880.5	8893.4	EIFNMAR
71	885.6	2675.6		885.4	1879.5	
72	888.5	7856.8	FDK(+94)YK(+94)			
73	891.5	4603.6	HDAHLNGK			
74				894.5	23864.3	
75	896.6	3000.5		896.5	5342.8	
76	898.8	2709.9				
77	903.8	3507.9	EKLTALTK/NIK(+76)IPSR			
78	906.6	48791.1	KYRAALGK/HFEK(+76)NR			
79	907.7	17470.7	TPALHFK(+94)/WK(+76)NEVR	907.5	5156.0	
80	908.6	3985.7	SLWDFLK			
81				909.5	2468.8	GMALFGEGK
82				913.6	33577.0	FFGEGTKK
83				915.6	9643.7	SKEVPEAR
84				916.5	8360.4	
85	918.3	4445.7	DLK(+94)NIK(+94)	918.4	3102.8	
86	919.4	5838.8		919.4	5254.2	
87	922.7	8486.1	NYQLYK(+94)/LGNNPVSK(+94)			
88				923.5	6077.8	QSFDLSVK
89	925.6	3368.4	WK(+94)NEVR			
90	927.7	28294.4	QH(+156)LFVK/LHVAGNLK(+76)			
91	928.8	7413.3	KLVKEALK/QLKEMK(+76)			
92				935.6	11137.1	TGLKEFLK
93				942.6	2548.0	IKDYFEK
94	947.7	3694.2				
95				950.5	1431.1	FVTQAEGAK
96				973.6	2037.5	YYELEEK/QIDDIDVR
97				983.6	23953.4	MGLAFESTK
98	990.8	10243.1	EERK(+94)IK(+94)			
99				991.6	5642.3	SLHMYANR
100	997.6	10191.8	QELNGNTK(+94)			
101				999.6	27493.4	SVGFHLPSR
102	1001.7	170062.0				
103	1003.9	43056.6				
104				1005.6	5928.5	
105	1011.3	1473.3	TK(+94)GTFAHR			
106				1012.6	2908.0	TGISPLALIK
107	1014.8	6185.4	KH(+16)VAEAICK			
108	1017.8	7907.0	LATALSLSNK/GDVKGSVLSR/QSFDLSVK(+94)/			
109			VK(+76)YTLNK(+76)			
110				1021.6	15179.0	NNALDFVTK
111				1022.2	1245.2	
112	1023.8	49029.0	QVFLYPEK			
113	1024.8	66719.2				
114	1029.8	1499.1	ALLK(+76)K(+76)TK(+76)	1029.4	1047.5	
115	1030.2	3033.5		1030.3	8008.7	
116	1031.3	6384.1	SISAALEHK(+76)	1031.2	9713.9	
117	1033.8	12066.0		1033.6	3119.5	
118	1036.2	51622.8	GAYQNNEIK	1036.1	13490.5	
119	1037.3	51294.3	YQK(+76)DK(+76)K(+76)	1037.2	14035.9	
120				1039.6	7378.4	LAPGELTIIL

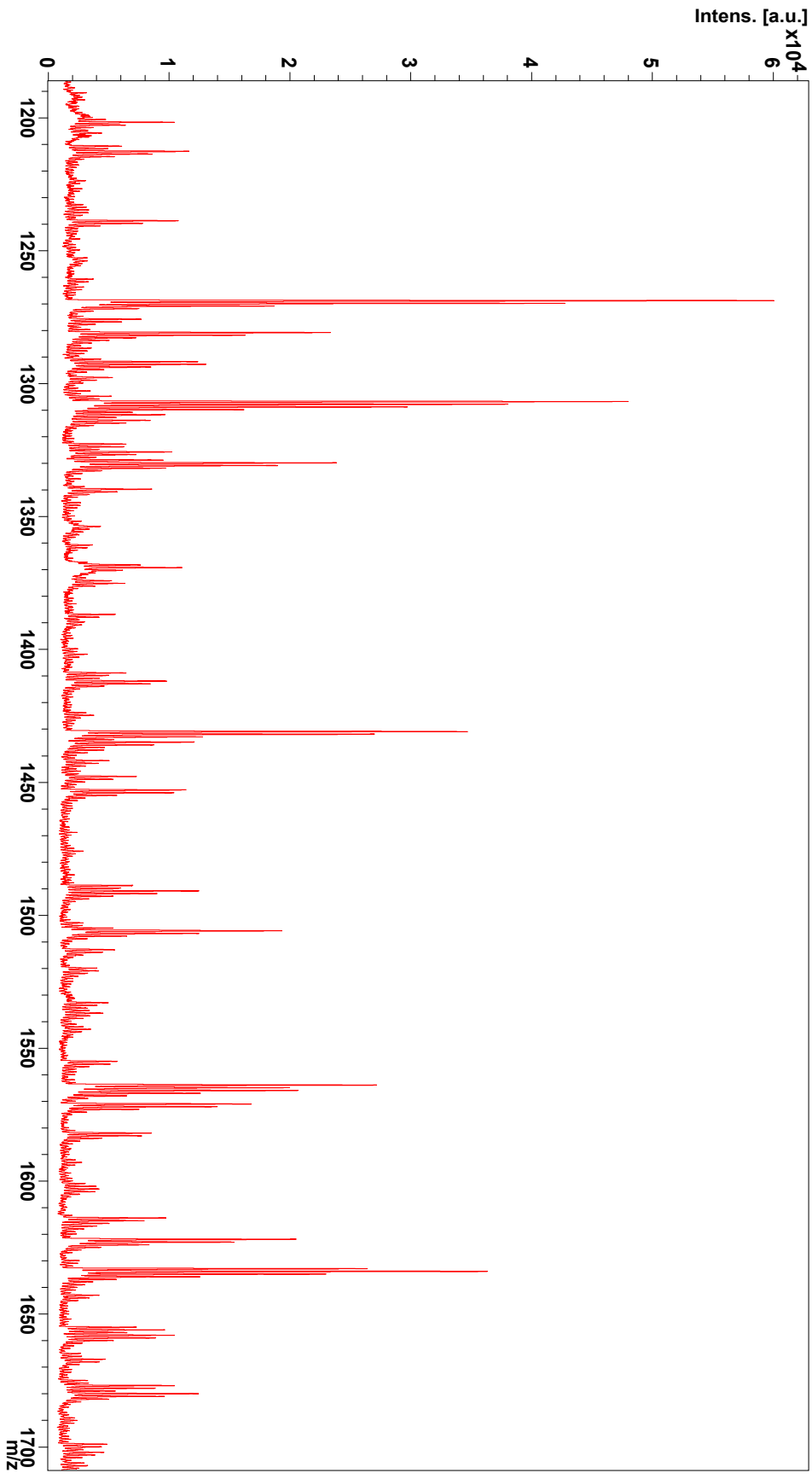
121	1042.8	14481.4	LKFIIPSPK	1042.6	1324.9	LKFIIPSPK
122				1043.6	4585.4	
123				1045.7	18087.6	IPSVQINFK
124				1047.6	4300.6	FPEVDVLTK
125	1053.2	2763.3				
126				1056.6	1015.5	QTEATMTFK
127	1058.8	4504.3	VQGVEFSHR/K(+76)YRAALGK(+76)/IK(+76)GVISIPR			
128				1061.7	5468.0	
129				1067.7	3139.2	
130	1068.6	5535.3	EVSSK(+94)LRR			
131				1070.7	17961.7	IEIPLPFGGK
132	1072.7	29624.6	YKNFATSNK/LTLDIQNKK/VNDESTEGK(+94)			
133				1076.6	2220.9	LVGFIDDAVK
134	1081.8	2058.1	LAAYLMLMR	1081.7	10605.8	LAAYLMLMR
135	1083.8	8934.3	ENLCLNLHK/KSISAALEHK/ALLK(+94)K(+94)TK(+94)			
136	1088.7	15803.8	TGISPLALIK(+76)	1088.7	10505.8	
137	1090.7	8690.2	GSWKWACPR			
138				1091.1	6337.9	
139	1094.7	31013.5	VPQTDMTFR/AKVHELIER/IK(+76)DYFEK(+76)			
140			K(+94)YRAALGK(+94)/			
141	1107.7	14822.7	AQYK(+76)K(+76)NK(+76)			
142				1110.6	1900.0	KIKGVISIPR
143				1111.7	2714.6	KMGLAFESTK
144				1113.7	3135.4	
145				1120.6	1684.3	LTIFKTELR
146	1129.7	8849.2				
147	1137.9	2442.4				
148	1138.7	44556.9	QGFFPDSVNK	1138.6	33912.9	QGFFPDSVNK
149				1141.7	20217.5	EELCTMFIR/HINIDQFVR
150	1142.9	54366.1	INDVLEHVK(+76)			
151	1149.7	3183.6				
152				1156.7	42945.8	SPAFTDLHLR
153				1160.6	10818.0	
154	1163.9	22455.1	DK(+76)IGVELTGR			
155	1164.9	47111.8	IEIPLPFGGK(+94)			
156	1166.7	24652.9	FRETLEDTR/K(+94)LNELSFK(+94)/GSWK(+76)WACPR	1166.7	3752.9	FRETLEDTR
157	1168.8	1765.3	NIILPVYDK(+94)	1168.7	4401.7	
158				1176.6	1323.2	VAWHYDEEK/GNVATEISTER
159	1177.9	4776.3	ENLCLNLHK(+94)			
160	1178.8	35551.9	VLVDHFGYTK	1178.7	10905.3	VLVDHFGYTK
161	1184.0	3282.5	DLKNIKIPSR			
162	1185.8	11644.0	EVK(+94)IDGQFR			
163	1188.7	2096.4	AQYKKNKH(+16)R/			
164			AK(+94)VHELIER			
165	1193.9	61054.7				
166	1195.8	119605.0	FLDMLIK(+94)K(+94)			
167	1197.9	11917.8	AEKSH(+16)DELPR			
168	1200.9	2322.1				
169				1201.7	7187.2	LTISEQNIQR
170				1205.7	1479.8	DEPTYILNIK
171				1210.7	3683.9	
172				1212.6	9380.0	NSEEFAAAMSR
173	1215.9	5126.6	EVYGFNPEGK(+76)			
174	1217.8	19814.8		1234.7	1190.2	
175	1236.9	5675.9	QH(+156)IEAIDVR			
176				1238.7	8809.4	YEGLQEWEGK/SEILAHWSPAK
177	1249.9	2715.0				
178	1252.8	1836.9	VAWHYDEEK(+76)			
179	1257.8	1206.8	H(+156)KFLDSNIK/AEK(+76)SHDELPR			
180				1260.7	1354.2	YELKLAIPEGK
181				1268.8	58592.2	DLKVEDIPLAR
182	1274.9	1042.5	NMEVSVATTTK(+94)			
183				1275.8	5936.9	LIVAMSSWLQK
184				1280.8	21146.7	SDGRVKYTLNK/TEVIPPLIENR
185	1283.9	15359.6	QH(+156)LFVKEVK			
186	1291.8	989.2	NRNNALDFVTK/LIVAMSSW(+16)LQK	1291.8	11420.5	NRNNALDFVTK
187	1297.8	1445.9	H(+156)INIDQFVR/EELC(+156)TMFIR	1297.7	2003.1	
188				1304.8	2226.1	KGNVATEISTER
189	1305.9	33839.7				
190				1306.8	47102.3	IISDYHQQFR/EFQVPTFTIPK
191				1308.8	12763.5	GFEPTLEALFGK
192	1311.8	3241.4	YFHK(+76)LNIPK(+76)	1311.7	4732.8	
193	1313.8	1239.6	NTFTLSCDGSLR/VLADKFIIPGLK/EK(+94)LTALTK(+94)K(+94)	1313.8	3962.0	NTFTLSCDGSLR/VLADKFIIPGLK
194				1322.8	4086.7	ESQLPTVMDFR/NPNGYSFSIPVK
195	1325.8	7025.8		1325.7	6461.7	
196	1327.9	31171.8	SKPTVSSSMEFK			
197				1328.8	5382.9	
198				1329.8	22047.1	KLTISEQNIQR
199	1331.9	1089.3	INSRFFGEGTK(+76)			
200	1339.8	8744.9	DFSLW(+16)EKTGLK/IYSLWEHSTK(+76)	1339.7	5538.9	
201	1349.9	2884.4	INSRFFGEGTK(+94)			
202	1353.8	2686.6		1353.6	1146.9	
203	1368.2	5156.2		1368.2	5193.2	
204				1369.2	6479.2	IQIQEKLQQLK
205	1373.2	2895.3				
206	1374.2	14848.5		1374.1	3046.3	
207	1375.3	8184.6				
208				1386.9	2312.2	IAELSATAQEIIK
209	1389.0	10130.8				
210	1401.9	15441.4	IPSVQINFKDLK/EH(+156)NAKFSLDGK			
211				1408.8	3715.6	EQHLFLPFSYK
212				1411.9	7108.2	QTIIVVLENVQR
213	1419.9	48987.6	IYSLWEH(+156)STK			
214	1423.9	12160.5	MNFKQELNGNTK/IVSLIKNLLVALK/K(+94)LTISEQNIQR/	1423.8	942.6	MNFKQELNGNTK/IVSLIKNLLVALK
215			DLK(+76)SK(+76)EVPEAR/DATRFK(+76)HLRK(+76)			
216				1430.9	31651.5	ALVEQGFTVPEIK
217	1431.0	2559.5	ALVEQGFTVPEIK			
218				1434.9	8527.5	KIISDYHQQFR/SSRDLKMLETVR
219	1440.1	12615.2				
220	1441.9	35002.6	TKNSEEFAAAMSR/TTK(+94)QSFDLSVK(+94)/AK(+76)FRETLEDTR	1441.8	2363.2	TKNSEEFAAAMSR
221	1444.9	5628.3	YH(+16)WEH(+16)TGLTLR			
222	1445.9	15919.4	DLSK(+76)EAQEVFK(+76)			
223	1447.8	3166.9	DLGQCDRFKPIR	1447.8	4106.0	DLGQCDRFKPIR
224				1452.9	9394.7	LNGEIQALELPQK/LDFREIQIYKK
225	1463.8	12416.1				
226	1465.9	14812.9	VLADK(+76)FIIPGLK(+76)			
227	1468.0	1797.3	GLK(+76)LATALSLSNK(+76)			
228	1480.0	77339.8	SK(+76)PTVSSSMEFK(+76)			
229	1485.8	4199.5	VNDESTEGKTSYR			
230	1488.0	2042.3				
231				1488.8	5649.3	AHLDIAGSLEGHLR
232				1490.8	8598.6	EAQEVFKAIQSLK
233	1502.0	6295.1	VLADKFIIPGLK(+94)			
234				1502.9	1838.0	HDAHLNGKVIGTLK/KHKLIDVISMYR
235	1505.1	21123.8	LK(+94)SLDEHYHIR/FQFPGK(+94)PGIYTR			
236				1505.8	15559.2	IGQDGISTSATTNLK
237	1512.1	1660.4	DFSLWEK(+94)TGLK(+94)/ANLFNK(+94)LVTELR			
238				1512.9	3452.9	NSLKIEIPLPFGGK
239				1519.9	2623.4	
240	1527.1	13074.1	YDFNSSMLYSTAK/ESQLPTVMDFRK(+76)/			

241			AGK(+76)LK(+76)FIIPSPK(+76)			
242	1533.0	26714.9		1532.9	2984.8	
243				1535.8	1952.2	
244	1538.0	2246.9	ATVAVYLESLQDTK/K(+94)QHLFVK(+94)EVK(+94)			
245				1541.9	1510.4	QSFDLSVKAQYKK
246	1555.0	23319.5	MDMTFSKQNALLR/TLQGIPQMIGEVIR	1555.0	4277.5	MDMTFSKQNALLR/TLQGIPQMIGEVIR
247	1563.9	12911.6	FLDSNIKFSHVEK	1563.9	23457.1	FLDSNIKFSHVEK
248				1565.9	9514.0	AVSMPSFSILGSDVR
249	1568.0	25023.4	YNALDLTNNGK(+76)LR			
250				1571.0	14864.7	GMALFGEGKAEFTGR
251	1574.0	40310.6	IK(+76)FDK(+76)YKAEK(+76)			
252	1577.1	4845.2				
253				1581.9	7207.9	HIYAISSAALSASYK
254	1590.0	2944.3	IPSVQINFK(+94)DLK(+94)/VK(+94)YTLNK(+94)NSLK(+94)/			
255			LLK(+76)ENLCLNLHK(+76)			
256				1593.0	778.6	LIDVISMYRELLK
257	1596.1	4488.2				
258				1600.9	1602.0	
259				1603.0	1943.3	VPQTDMTFRHVGSK/LALWGEHTGQLYSK
260				1613.9	7861.9	MTSNFPVDLSDYPK
261				1617.0	1172.5	TSSFALNLPTLPEVK
262	1621.1	7172.7	YDFNSSMLYSTAK(+94)			
263				1621.9	17255.6	NFATSNKMDMTFSK
264	1622.1	26687.7	NFATSNKMDMTFSK			
265	1626.9	2219.6	YYELEEKIVSLIK			
266	1633.2	1870.6	ALYWVNGQVPDGVSK/EK(+94)LTALTK(+94)K(+94)YR	1632.9	30469.0	ALYWVNGQVPDGVSK
267				1635.0	7141.9	
268				1636.0	3869.6	LDFSSQADLRNEIK
269	1640.1	131397.7				
270				1642.9	2208.7	AASGTTGTYQEWKDK/RHIQNIDIQHLAGK
271	1644.4	2954.1	QHLRVSTAFVYTK(+94)/LDNIYSSDK(+76)FYK(+76)			
272						
273	1646.1	6348.4	IK(+94)FDK(+94)YK(+94)AEK(+94)			
274				1654.9	7232.8	
275	1655.3	2824.2	SYNETK(+94)IK(+94)FDK(+94)/NTFTLSCDGSLRHK(+76)/			
276			K(+76)HK(+76)LIDVISMYR/HDAHLNGK(+76)VIGTLK(+76)			
277				1658.0	7474.7	SVSDGIAALDLNAVANK
278	1662.1	20609.3				
279				1664.9	1120.3	
280				1667.0	2422.3	GIISALLVPPETEEAK
281				1675.0	1588.5	SDTNGKYKNFATSNK
282				1676.9	8609.0	
283				1680.0	8493.1	
284	1693.1	1461.0	EVYGFNPEGKALLKK/VLVDH(+156)FGYTKDDK/			
285			TSSFALNLPTLPEVK(+76)/K(+76)LQSTTVMNPYMK(+76)			
286				1699.0	2952.7	
287				1702.0	2363.8	ATLYALSHAVNNYHK
288				1706.1	1411.7	NIQEYLSILTDPDGK/RQHLRVSTAFVYTK
289	1712.2	1529.6	INAFRAKVH(+16)ELIER/FSH(+156)VEKLGNNPVSK/			
290			ITEVALMGHLSCDTK(+94)/VELEVPQLCSFILK(+94)/			
291			NIFNFK(+94)VSQEGLK(+94)/LDFSSQADLRNEIK(+76)			
292	1715.1	1929.6				
293				1717.0	5411.2	LNIPKLDFSSQADLR
294	1724.0	4578.9	IVQILPWEQNEQVK	1724.1	32607.9	IVQILPWEQNEQVK
295	1726.1	3081.8	KIISDYHQQFRYK/TLLKAGH(+156)IAWTSSGK			
296	1730.2	1897.4	HEQDMVNGIMLSVEK/LDFSSQADLRNEIK(+94)/			
297			IEFEWNTGTNVDTK(+76)			
298				1738.0	925.2	
299				1742.0	2330.5	KMTSNFPVDLSDYPK
300				1746.0	14219.7	NSEEFAAAMSRYELK/KITEVALMGHLSCDTK
301	1751.1	3526.5	LQDFSDQLSDYYEK/LK(+76)FIIPSPKRPVK(+76)			
302				1756.0	959.2	
303				1764.0	6447.8	EALKESQLPTVMDFR
304				1769.0	1464.7	
305	1773.1	2364.0				
306				1776.0	27497.3	NLQNNAEWVYQGAIR
307				1780.0	1410.7	
308	1796.1	1275.3	DNVPKATGVLYDYVNK/ATLYALSHAVNNYHK(+94)/			
309			EK(+76)IAELSATAQEIIK(+76)			
310				1798.1	988.3	
311				1834.1	28442.0	ATFQTPDFIVPLTDLR
312				1856.1	758.4	
313	1863.0	1034.1	FDKYKAEKSHDELPR			
314				1874.2	3448.6	EVKIDGQFRVSSFYAK/FSVPAGIVIPSFQALTAR
315	1879.0	1692.4	ALVDTLK(+94)FVTQAEGAK(+94)			
316	1881.0	42000.3	AAIQALRK(+94)MEPK(+94)DK(+94)/			
317			QAEAVLK(+94)TLQELK(+94)K(+94)			
318				1883.1	2086.6	
319				1889.1	4248.9	VIGNMGQTMEQLTPELK/LTISEQNIQRANLFNK
320	1902.0	4545.6	KITEVALMGH(+156)LSCDTK/KITEVALMGHLSC(+156)DTK			
321				1905.1	595.4	
322				1907.1	2434.6	LQAEARSEILAHWSPAK
323	1908.0	3454.9	LIK(+94)DLK(+94)SK(+94)EVPEAR			
324				1911.1	1433.9	
325				1921.1	712.9	
326				1923.0	908.5	
327	1924.0	4114.1	EYSGTIASEANTYLNSK(+76)			
328	1928.1	854.2				
329				1929.1	2552.8	NLTDFAEQYSIQDWAK/YQIRIQIQEKLQQLK
330	1930.1	2839.8	FEVDSPVYNATW(+16)SASLK/K(+94)MTSNFPVDLSDYPK(+94)			
331				1932.1	516.7	RNLQNNAEWVYQGAIR
332	1946.1	9572.3				
333				1947.2	3345.0	SPAFTDLHLRYQKDKK/ATLRIGQDGISTSATTNLK/TILGTMPAFEVSLQALQK
334	1956.1	2055.1				
335				1963.2	2614.9	
336	1969.1	663.8	TIHDLHLFIENIDFNK	1969.2	1032.9	TIHDLHLFIENIDFNK
337	1978.1	2558.6	TFIEDVNK(+76)FLDMLIK(+76)			
338				1985.2	581.2	NNALDFVTKSYNETKIK
339				1995.1	4966.5	LPQQANDYLNSFNWER/ALLKKTKNSEEFAAAMSR
340	2000.0	882.7	IKDYFEKLVGFIDDAVK/QH(+156)LFVKEVKIDGQFR			
341				2014.1	10774.9	SFDYHQFVDETNDKIR/NRNNALDFVTKSYNETK/KLRTSSFALNLPTLPEVK
342	2017.2	2101.5	KLTISEQNIQRANLFNK			
343	2020.1	4181.8	IEDGTLASK(+94)TK(+94)GTFAHR			
344				2022.2	906.3	LAAYLMLMRSPSQADINK/YGMVAQVTQTLKLEDTPK
345				2036.1	474.0	LVGFIDDAVKKLNELSFK/LIDVISMYRELLKDLSK
346	2042.0	2775.4	YKLQDFSDQLSDYYEK/TLQELKKLTISEQNIQR	2042.1	977.3	YKLQDFSDQLSDYYEK/TLQELKKLTISEQNIQR
347	2045.1	994.2	H(+16)INIDQFVRKYRAALGK/TIHDLHLFIENIDFNK(+76)			
348				2053.3	2172.4	ILGEELGFASLHDLQLLGK
349				2073.2	1894.7	ASGSLPYTQTLQDHLNSLK
350				2089.2	456.7	
351				2147.2	2228.2	DQEVLLQTFLDDASPGDKR
352				2154.2	666.1	YTYNYEAESSSGVPGTADSR/TTLTAFGFASADLIEIGLEGK
353	2163.1	2219.4	TK(+94)NSEEFAAAMSRYELK(+94)	2163.3	22141.1	
354	2185.1	2811.7	H(+156)INIDQFVRKYRAALGK	2185.3	6377.2	
355				2190.3	450.1	EAQEVFKAIQSLKTTEVLR
356				2203.3	403.0	
357				2210.4	2197.9	QVFLYPEKDEPTYILNIK/IPSVQINFKDLKNIKIPSR
358				2234.3	659.6	DKDQEVLLQTFLDDASPGDK
359				2243.5	949.1	VPSYTLILPSLELPVLHVPR
360				2251.2	940.1	

361				2262.4	796.9	DNVFDGLVRVTQEFHMKVK/LPYTIITTPPLKDFSLWEK
362				2273.4	10344.2	
363	2277.1	1432.8	ALLK(+94)K(+94)TK(+94)NSEEFAAAMSR			
364				2282.3	1871.3	KYTYNYEAESSSGVPGTADSR/EALKESQLPTVMDFRKFSR
365				2289.4	966.5	
366	2295.1	1340.5	LEDTPK(+76)INSRFFGEGTK(+76)K(+76)	2295.4	2087.6	
367	2299.0	1386.6	YLRTEHGSEMLFFGNAIEGK/			
368			EVYGFNPEGK(+94)ALLK(+94)K(+94)TK(+94)/			
369			HLRVNQNLVYESGSLNFSK(+94)			
370	2301.1	2098.2	HKLIDVISMYRELLKDLSK/			
371			IGVELTGRTLADLTLLDSPIK(+76)			
372				2366.5	769.3	AQNLYQELLTQEGQASFQGLK/QVFLYPEKDEPTYILNIKR
373				2390.4	20749.2	DKDQEVLLQTFLDDASPGDKR
374	2405.2	888.3	LQQLK(+76)RHIQNIDIQHLAGK(+76)			
375				2412.4	721.8	
376	2427.1	570.1	NSLK(+76)IEIPLPFGGK(+76)SSRDLK(+76)			
377				2431.4	1265.3	TIDQMLNSELQWPVPDIYLR
378				2435.4	1178.7	AALGKLPQQANDYLNSFNWER/LTIFKTELRVRESDEETQIK
379	2458.1	2062.9	DDKH(+16)EQDMVNGIMLSVEKLIK			
380				2466.6	4644.2	IADFELPTIIVPEQTIEIPSIK
381	2480.0	1695.5	ENFAGEATLQRIYSLWEHSTK/			
382			LSLESLTSYFSIESSTK(+94)GDVK(+94)/			
383			YFHK(+94)LNIPK(+94)LDFSSQADLR/			
384			IK(+94)FDK(+94)YK(+94)AEK(+94)SHDELPR			
385				2483.5	432.2	IDGQFRVSSFYAKGTYGLSCQR/NFVASHIANILNSEELDIQDLK
386				2488.6	1357.4	
387	2498.1	10005.4	ADSVVDLLSYNVQGSGETTYDHK			
388				2509.4	4055.0	DFSAEYEEDGKYEGLQEWEGK
389	2520.0	7162.6				
390	2529.1	7080.1	ALLKKTKNSEEFAAAMSRYELK/KITEVALMGHLSCDTKEERKIK/			
391			AALGK(+94)LPQQANDYLNSFNWER/			
392			HK(+76)LIDVISMYRELLK(+76)DLSK(+76)			
393			FLDSNIKFSH(+156)VEKLGNNPVSK/			
394				2531.4	906.8	NEVRIHSGSFQSQVELSNDQEK/HFVINLIGDFEVAEKINAFRAK
395	2541.1	3850.4	DSYDLHDLKIAIANIIDEIIEK/			
396			SLWDFLKLDVTTSIGRRQHLR/			
397			GSVLSREYSGTIASEANTYLNSK(+94)/			
398			NLKH(+156)INIDQFVRKYRAALGK			
399				2550.5	3936.1	LIDLSIQNYHTFLIYITELLK
400	2551.1	1637.8	LIDLSIQNYHTFLIYITELLK/ETIQKLSNVLQQVKIKDYFEK/			
401			LFLEETK(+76)ATVAVYLESLQDTK(+76)			
402				2560.6	630.1	
403	2563.0	657.4	KLTIFKTELRVRESDEETQIK/			
404			TTKQSFDLSVKAQYKKNKH(+156)R/			
405			VSSFYAKGTYGLSC(+156)QRDPNTGR/			
406			GMTRPLSTLISSSQSCQYTLDAK(+76)/			
407			VNDESTEGK(+76)TSYRLTLDIQNK(+76)			
408				2572.5	1106.5	
409				2581.5	819.1	LQDFSDQLSDYYEKFIAESKR
410	2587.1	567.6	AEPLAFTFSHDYKGSTSHHLVSR/			
411			LFLEETK(+94)ATVAVYLESLQDTK(+94)/			
412			LTIFK(+76)TELRVRESDEETQIK(+76)			
413				2592.5	1183.1	EYSGTIASEANTYLNSKSTRSSVK
414				2611.7	749.5	NFVASHIANILNSEELDIQDLKK
415				2645.7	1223.7	LPQQANDYLNSFNWERQVSHAK/NRNNALDFVTKSYNETKIKFDK/TSSFALNLPTLPEVKFPEVDVLTK
416	2867.2	3203.5	LIDVISMYRELLKDLSKEAQEVFK/			
417			LIDLSIQNYHTFLIYITELLK(+94)K(+94)/			
418			K(+76)MTSNFPVDLSDYPK(+76)SLHMYANR/			
419			GSVLSREYSGTIASEANTYLNSK(+76)STR/			
420			AEPLAFTFSHDYK(+76)GSTSHHLVSRK(+76)			
421	2889.1	1785.5	QTIIVVLENVQRNLKHINIDQFVR/			
422			NEIK(+94)TLLK(+94)AGHIAWTSSGK(+94)GSWK(+94)/			
423			KGNVATEISTERDLGQC(+156)DRFKPIR/			
424			LPYTIITTPPLK(+76)DFSLWEK(+76)TGLK(+76)			
425	2949.1	1223.4	ATGVLYDYVNKYH(+156)WEH(+156)TGLTLR/			
426			ALYWVNGQVPDGVSKVLVDH(+156)FGYTK/			
427			K(+94)SISAALEHK(+94)VSALLTPAEQTGTWK(+94)/			
428			NRNNALDFVTK(+76)SYNETK(+76)IK(+76)FDK(+76)/			
429			LEVLNFDFQANAQLSNPK(+76)INPLALK(+76)			
430	2997.7	1189.8	YNRQSMTLSSEVQIPDFDVDLGTILR/			
431			ITLIINWLQEALSSASLAH(+156)MKAKFR/			
432			TTEVLRNLQDLLQFIFQLIEDNIK(+94)/			
433			WK(+94)NEVRIHSGSFQSQVELSNDQEK(+94)/			
434			GDVK(+94)GSVLSREYSGTIASEANTYLNSK(+94)			
435				3235.0	293.8	
436				3441.3	481.6	YSQPEDSLIPFFEITVPESQLTVSQFTLPK/TLQELKKLTISEQNIQRANLFNKLVTELR
437	3574.2	1542.2	GLLIFDASSSWGPQMSASVH(+16)LDSKKKQH(+16)LFVK/			
438			LTRKRGLKLATALSLSNKFVEGSH(+16)NSTVSLTTK/			
439			LRRNLQNNAEW(+16)VYQGAIRQIDDIDVRFQK			

Table 3-3. Peaks in MALDI-TOF spectra of intact LDL (commercial) or *Mtb*-LDL and the matched sequences. The peaks which are the same in *Mtb*-LDL MALDI-TOF spectrum and LDL MALDI-TOF spectrum were indicated in red.


Intens. [a.u.]
x10^4
0
1
2
3
4
5
700
750
800
850
900
950
1000
1050
1100
1150
1200
m/z


Intens. [a.u.]
x10^4
0
1
2
3
4
5
6
1200
1250
1300
1350
1400
1450
1500
1550
1600
1650
1700
m/z

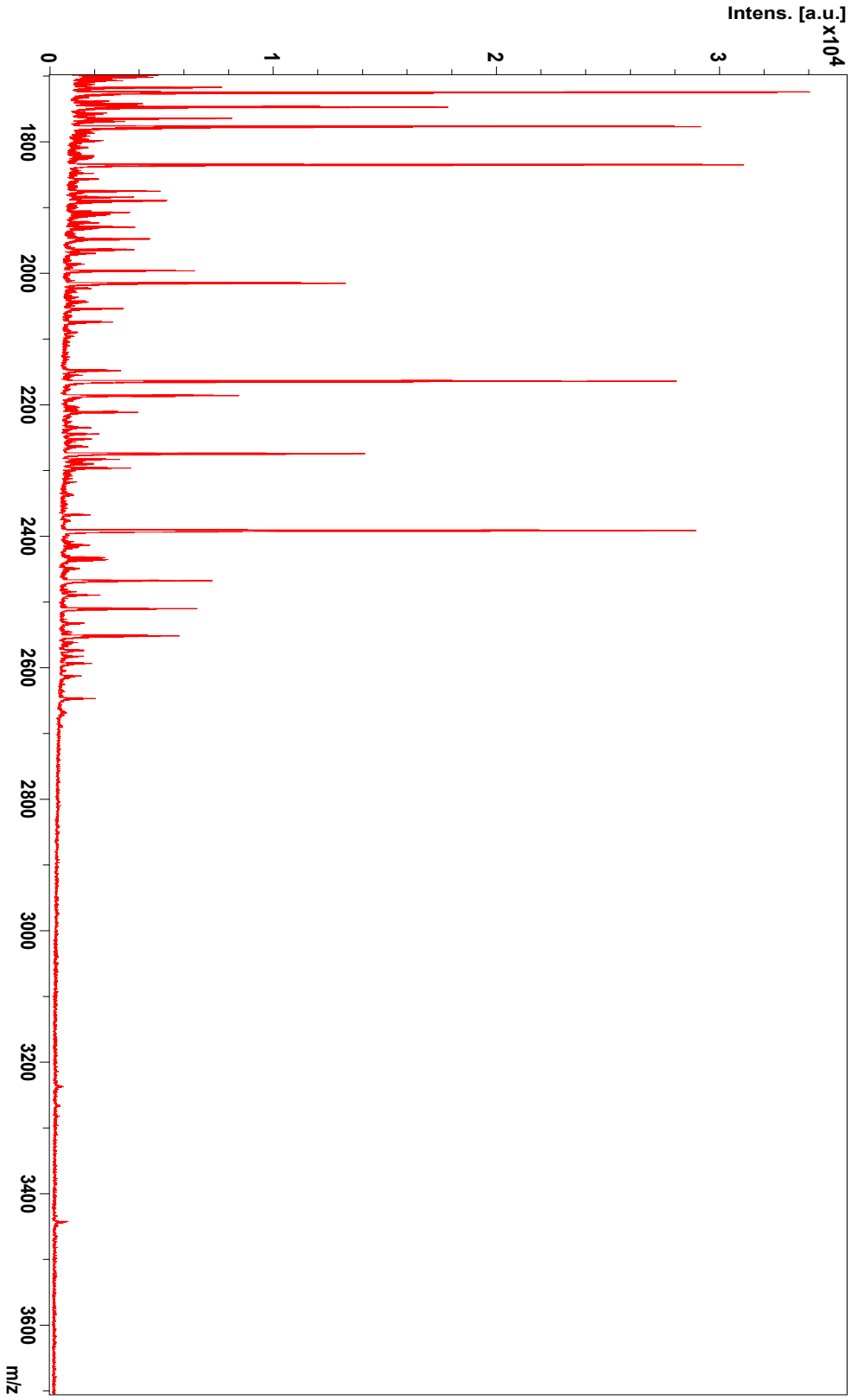
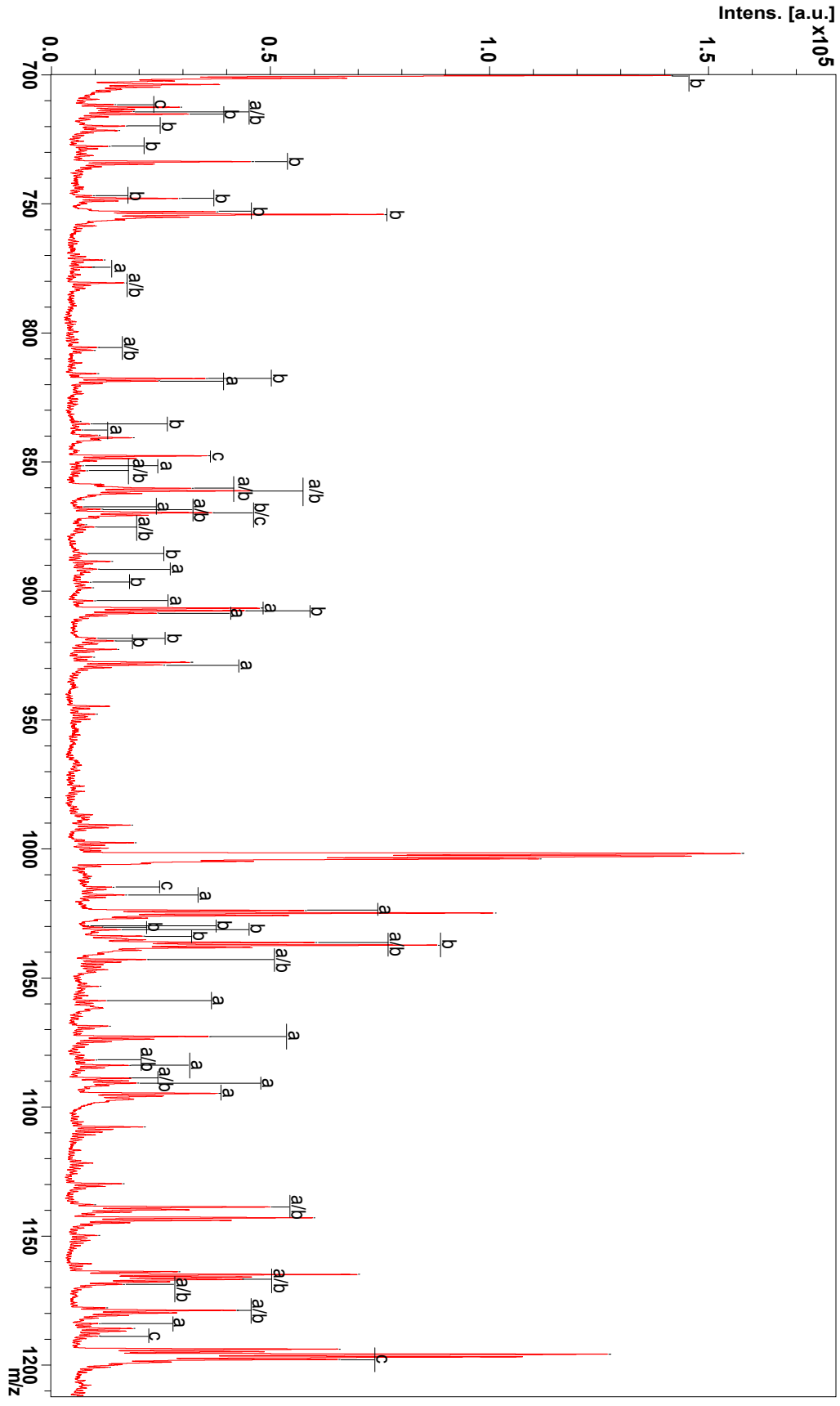
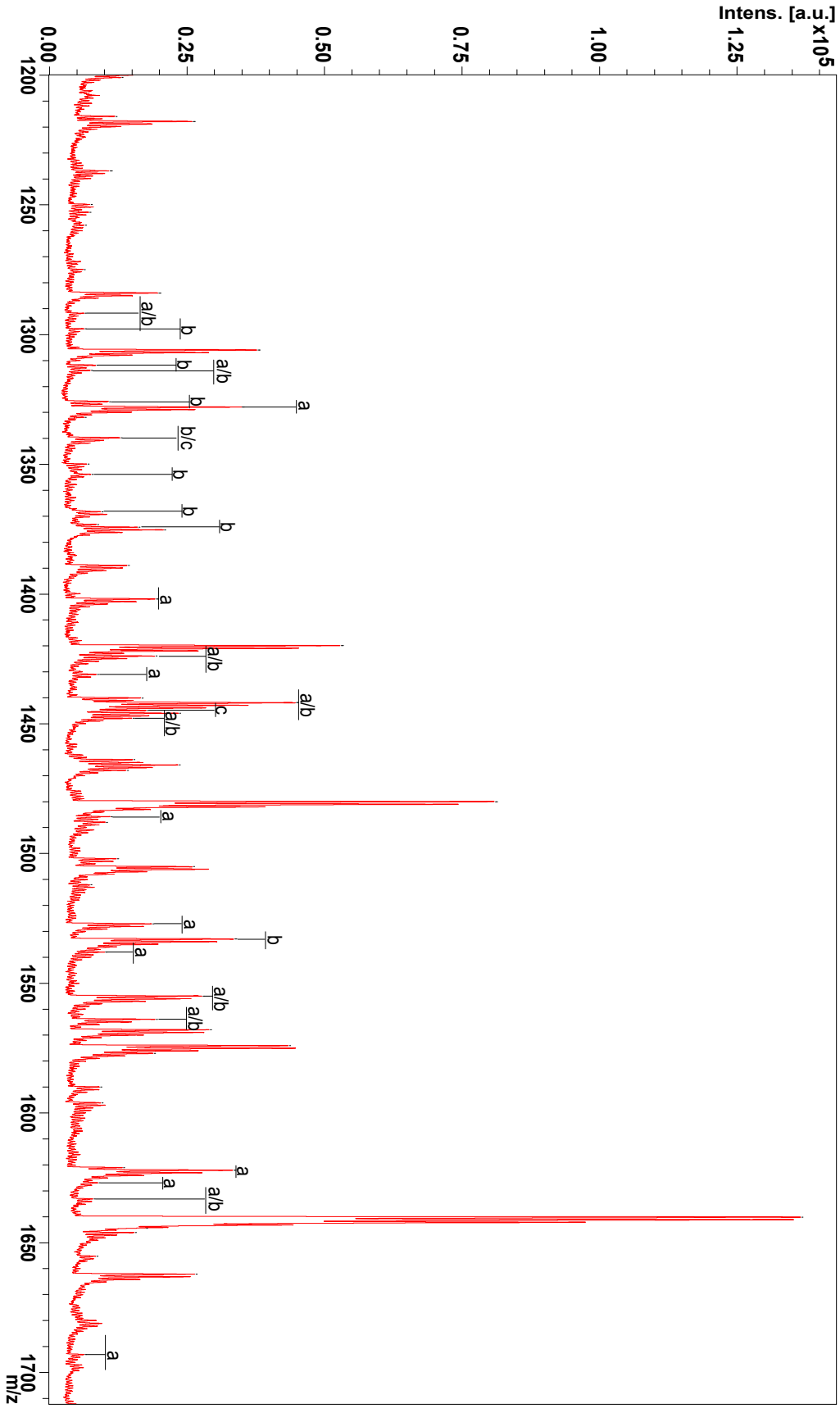


Figure 3-8. MALDI-TOF spectrum of intact LDL (commercial).


Intens. [a.u.]
x10^5
0.0
0.5
1.0
1.5
700
750
800
850
900
950
1000
1050
1100
1150
1200
m/z


Intens. [a.u.]
x10^5
0.00
0.25
0.50
0.75
1.00
1.25
1200
1250
1300
1350
1400
1450
1500
1550
1600
1650
1700
m/z
a/b
b
a/b
b
a
b/c
b
b
b
a
a/b
a
a/b
c
a/b
a
a
b
a
a/b
a/b
a
a
a/b
a

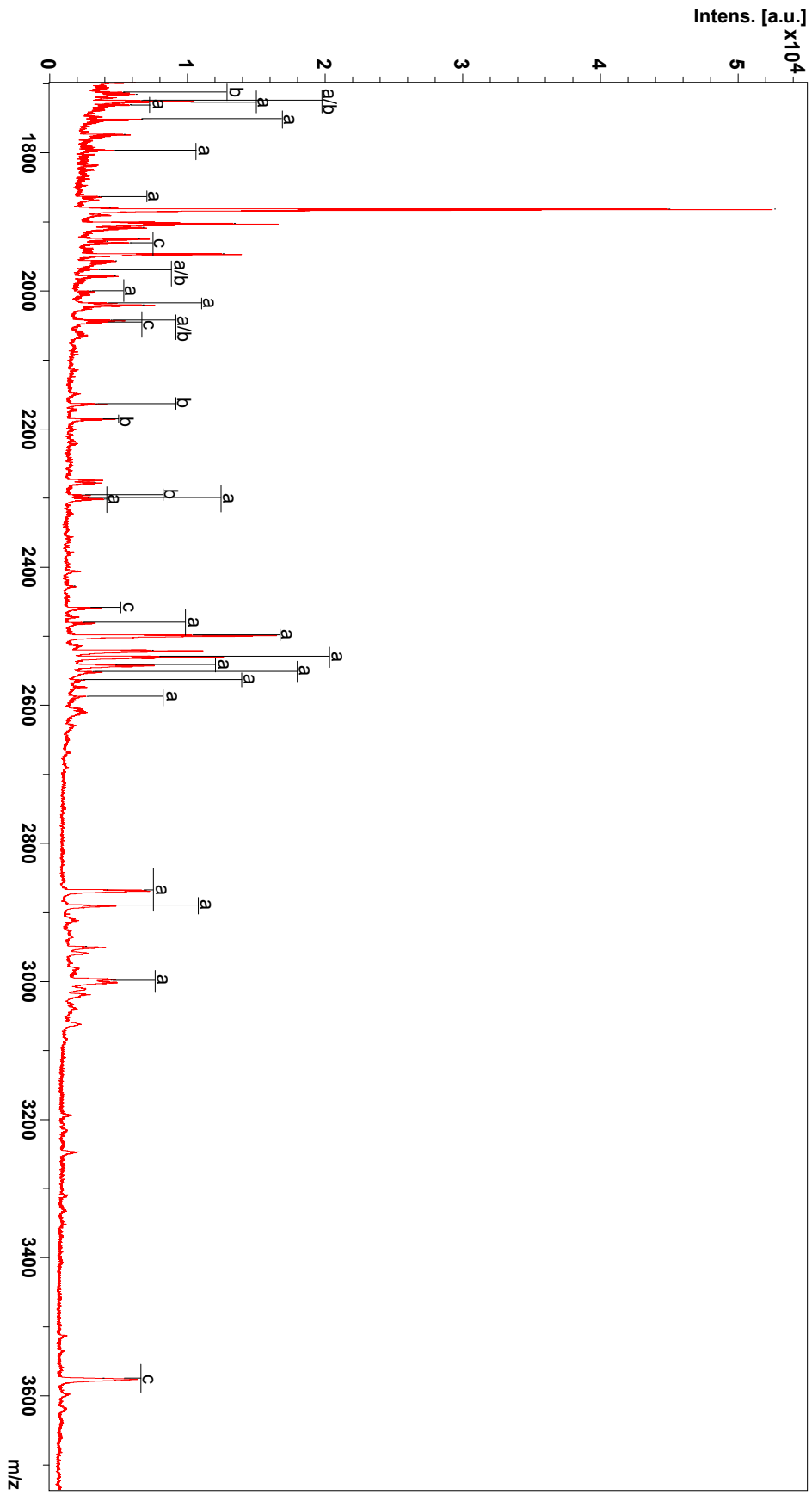
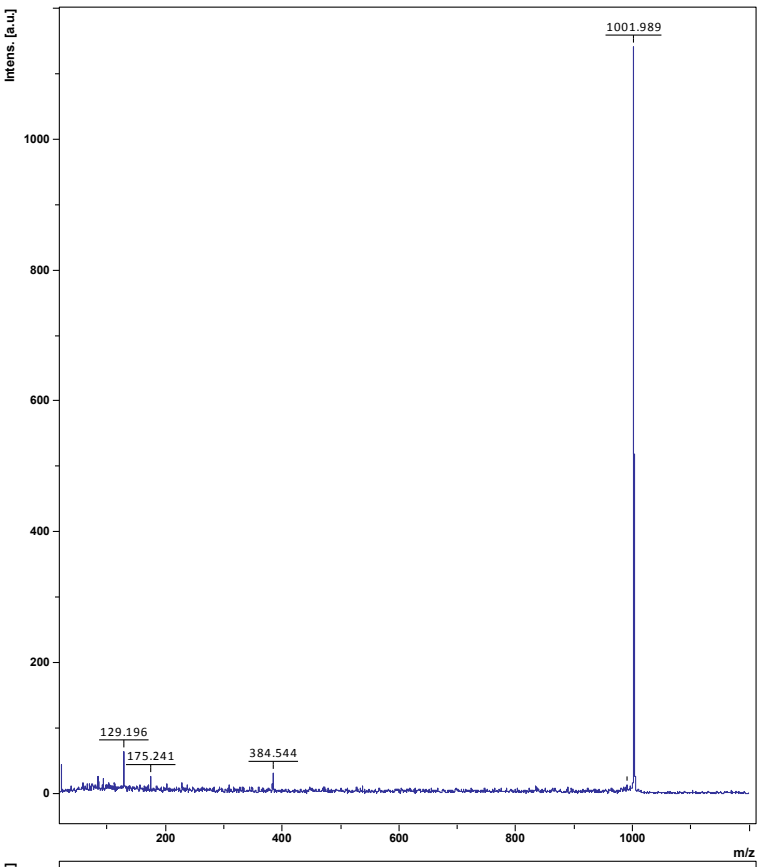
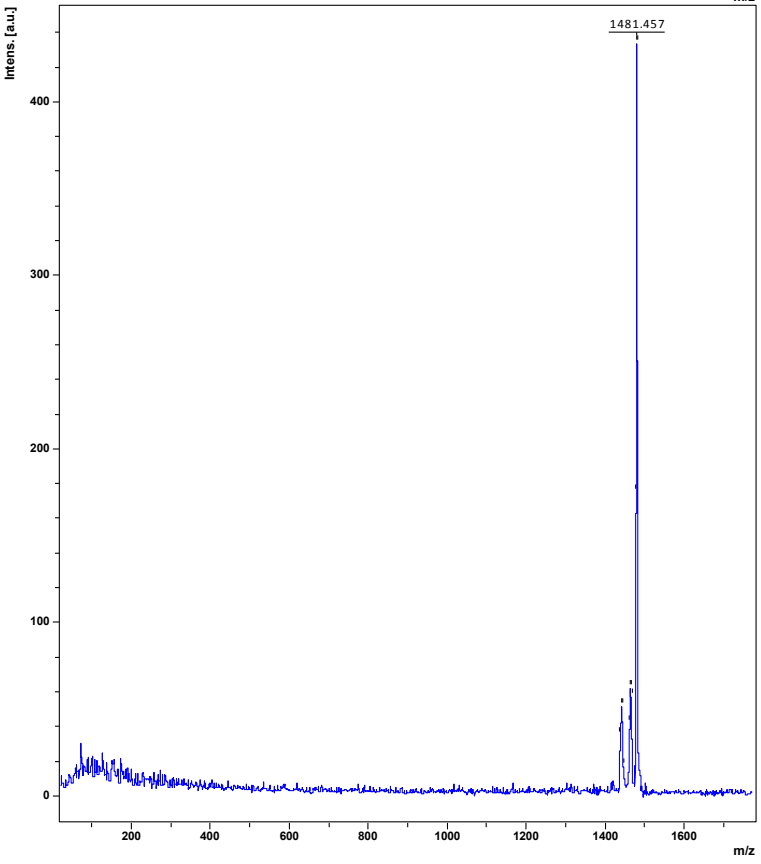

Intens. [a.u.]
x10^4
0
1
2
3
4
5
m/z
1800
2000
2200
2400
2600
2800
3000
3200
3400
3600

Figure 3-9. MALDI-TOF spectrum of *Mtb*-LDL. The peaks that are identified by matching with theoretically digested apoB100 are indicated with "a"; the peaks that match those of the intact LDL (commercial) spectrum are indicated with "b"; the peaks with potential oxidized His or Trp are indicated with c; the peaks that match those of both theoretically digested apoB100 and the intact LDL (commercial) spectrum are indicated with "a/b"; the peaks that match those of the intact LDL (commercial) spectrum and have potential oxidized His or Trp are indicated with "b/c".

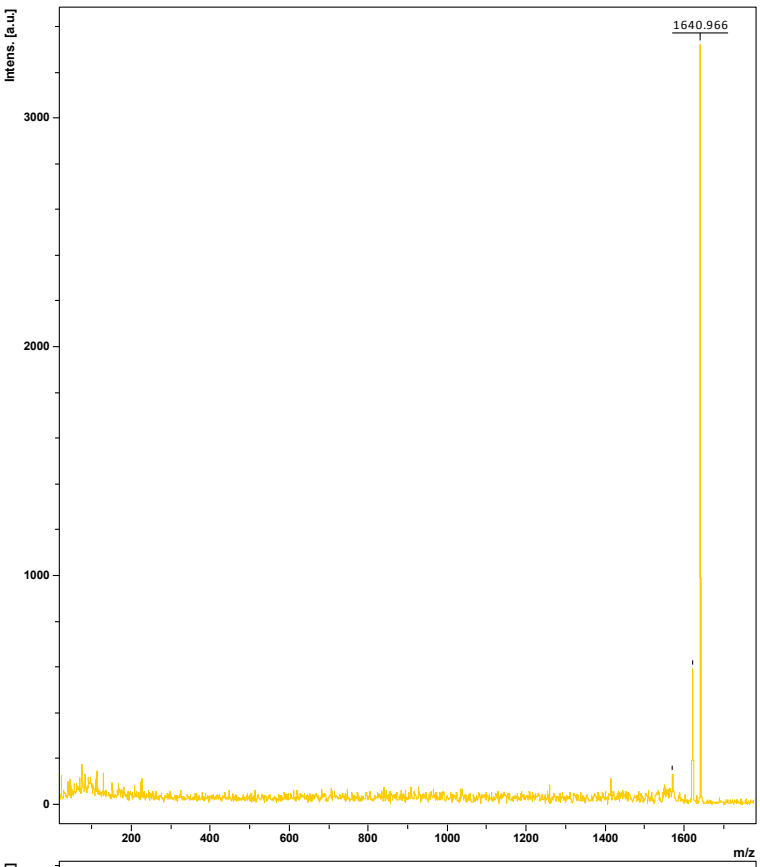
A.


Intens. [a.u.]
1001.989
129.196
175.241
384.544
m/z

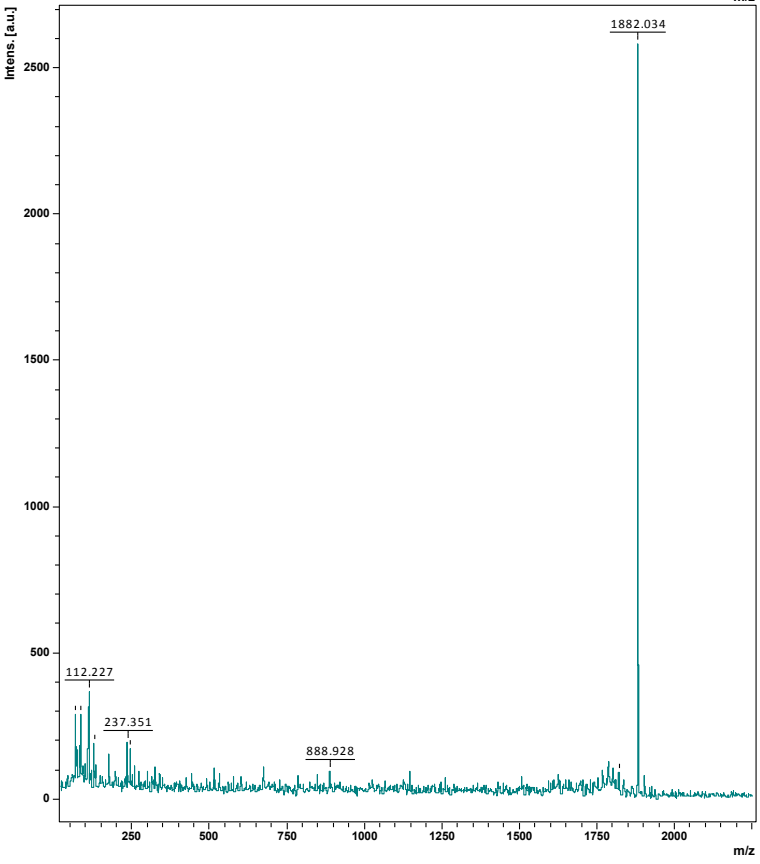
B.


Intens. [a.u.]
1481.457
m/z

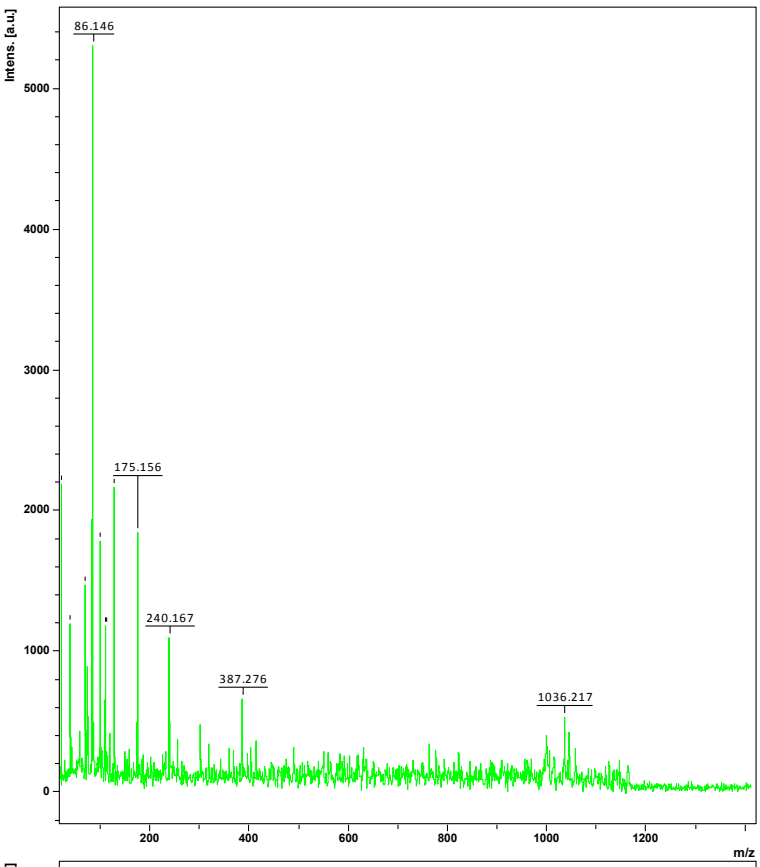
C.


Intens. [a.u.]
1640.966
3000
2000
1000
0
200
400
600
800
1000
1200
1400
1600
m/z

D.


Intens. [a.u.]
1882.034
2500
2000
1500
1000
500
0
112.227
237.351
888.928
250
500
750
1000
1250
1500
1750
2000
m/z

E.


Intens. [a.u.]
86.146
175.156
240.167
387.276
1036.217
m/z

F.

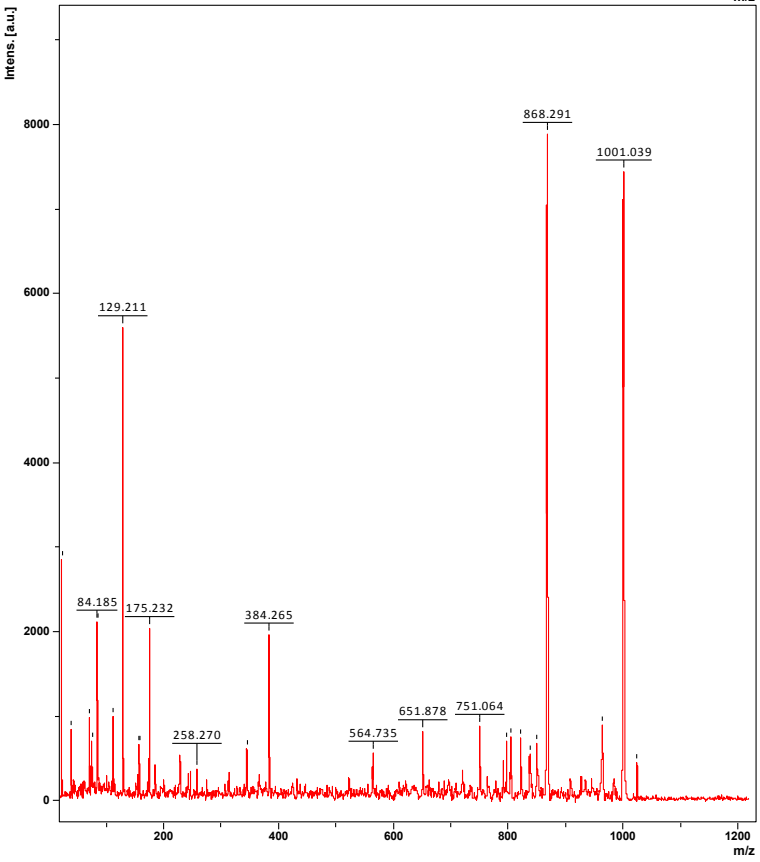

Intens. [a.u.]
868.291
1001.039
129.211
84.185
175.232
384.265
258.270
564.735
651.878
751.064
m/z

Figure 3-10. MALDI-TOF/TOF spectra for tryptic peptides from *Mtb*-LDL. (**A**) MALDI-TOF/TOF spectrum of the parent ion m/z 1001. (**B**) MALDI-TOF/TOF spectrum of the parent ion m/z 1481. (**C**) MALDI-TOF/TOF spectrum of the parent ion m/z 1640. (**D**) MALDI-TOF/TOF spectrum of the parent ion m/z 1882. (**E**) MALDI-TOF/TOF spectrum of the parent ion m/z 1036. (**F**) MALDI-TOF/TOF spectrum of the parent ion m/z 1024.

3.3. Conclusion and future directions

In this study, we successfully purified LDL from Hep G2 cells, prepared OxLDL, Acro-LDL, and *Mtb*-LDL. THP-1 macrophages were used to mimic human monocyte-derived macrophages. From macrophage experiment, we demonstrated that *Mtb*-LDL could cause foamy macrophage formation to a much higher degree compared with intact LDL, OxLDL and Acro-LDL. Using agarose gel electrophoresis, we found the charge and size of *Mtb*-LDL was different from that of intact LDL, OxLDL and Acro-LDL. *Mtb*-LDL particles were more positively charged, and the size was larger. Using Western blot analysis, we further demonstrated that *Mtb*-LDL was different from intact LDL, OxLDL and Acro-LDL, because anti-apoB antibody, anti-LDL (copper oxidized) antibody and anti-LDL (MDA oxidized) antibody could not recognize *Mtb*-LDL. Finally, an on-membrane tryptic digest experiment and MALDI-TOF analysis were carried out to analyze the modification on apoB100 of *Mtb*-LDL, and some potential modified peptides were found.

In the future, we will test serum from pediatric TB patients for the presence of *Mtb*-LDL. We still need to further analyze apoB100 from *Mtb*-LDL by using MS/MS to identify the modified peptide sequences. Also, using the *Mtb*-LDL, we will select antibodies specific for *Mtb*-LDL to undertake the diagnosis of TB from serum samples.

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