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Detection of MMPs and their Inhibitors Using a Whole Blood Protease Assay

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Bioengineering

by

Mrudul Vinayak Bhide

Committee in charge:

Professor Michael J. Heller, Chair
Professor Geert W. Schmid-Schönbein
Professor Karen L. Herbst

2012
The thesis of Mrudul Vinayak Bhide is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

(Chair)

University of California, San Diego

2012
DEDICATION

I would like to dedicate this thesis and degree to my parents, my brother and my sister for their unwavering support, love and kindness - I would not be here without them.
"Education is a progressive discovery of your own ignorance."

~ Will Durant
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LIST OF ABBREVIATIONS

ANOVA.................................................................Analysis of Variance

BFL.................................................................................. Bodipy FL

BMI................................................................................. Body Mass Index

BP.................................................................................. Blood Pressure

BRET.................................................................Bioluminescence Resonance Energy Transfer

c-JNK...........................................................................c-Jun NH2-terminal Kinase

COX-2...........................................................................Cyclooxygenase-2

CVD...........................................................................Cardiovascular Disease

DALY.................................................................Disability-Adjusted Life Year

DHA..............................................................................Docosahexaenoic Acid

DMSO..........................................................................Dimethylsulfoxide

ε.................................................................epsilon amino

EDTA.............................................................................Ethylenediaminetetraacetic Acid

ECM.............................................................................Extracellular Matrix

FA..................................................................................Fatty Acid

FDA.............................................................................Food And Drug Administration

FRET...........................................................................Fluorescence Resonant Energy Transfer
GI............................Glycemic Index
GSE..............................................Grape Seed Extract
IL-1.............................................Interleukin-1
IL-1β............................Interleukin-1 beta
IL-6.............................................Interleukin-6
LA............................................Linoleic Acid
LV..........................................Left Ventricular
MAPK..........................Mitogen-Activated Protein Kinase
MI.......................................Myocardial Infarction
MMP..................................Matrix Metalloproteinase
MMP-2..........................Matrix Metalloproteinase-2
MMP-9..........................Matrix Metalloproteinase-9
MMPI..........................Matrix Metalloproteinase Inhibitor
MT-MMP......................Membrane-Type Matrix Metalloproteinases
NF-κB....................Nuclear Factor kappa-light-chain-enhancer of activated B cells
PAGE..........................Polyacrylamide Gel Electrophoresis
PBS..........................Phosphate Buffered Saline
PI .................................................................Phosphoinositide
PKC ........................................................................Protein Kinase C
PPARγ ........................................Peroxisome Proliferators- Activated Receptor gamma
TBE .................................................................Tris Borate EDTA
TIMP ..................................................Tissue Inhibitor of Metalloprotease
TIMP-1 ..................................................Tissue Inhibitor of Metalloprotease-1
TIMP-2 ..................................................Tissue Inhibitor of Metalloprotease-2
TIMP-3 ..................................................Tissue Inhibitor of Metalloprotease-3
TIMP-4 ..................................................Tissue Inhibitor of Metalloprotease-4
TNF-α ...............................................................Tumor Necrosis Factor alpha
WHO .............................................................World Health Organization
YLD .................................................................Years Lost Due to Disability
YLL .................................................................Years of Life Lost Due to Premature Mortality
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ABSTRACT OF THE THESIS

Detection of MMPs and their Inhibitors Using a Whole Blood Protease Assay

by

Mrudul Vinayak Bhide

Master of Science in Bioengineering

University of California, San Diego, 2012

Professor Michael J. Heller, Chair

The level of Matrix Metalloproteinases (MMPs) in whole blood has been investigated, with an emphasis on the effect of certain MMP inhibitors. A synthetically derived charge changing substrate, which has a cleavage site specific to enzymes MMP-2 and MMP-9, was used for detection. Presence of these enzymes was tested both with and without inhibitors, first in a buffer solution, and then in whole blood. The blood was taken from insulin resistant volunteers at different time points after the consumption of a meal rich in protein and fat. The volunteers were part of a twelve-week study performed to assess the change in levels of MMPs in the blood after being on a low-glycemic index diet. The presence of these enzymes in blood was assessed by reacting the blood with the synthetic substrate for a fixed period of time,
and then using PAGE (polyacrylamide gel electrophoresis). After electrophoresis, the gel was imaged and enzyme levels quantified using a fluorescent detector. The gels were analyzed for intensity of fluorescence, which had a direct correlation with the amount of the MMP-2/9 present. Results indicated that there were slight changes in the levels of these enzymes after the 12-week period. However, the various MMP inhibitors such as grape seed extract (GSE), and doxycycline showed a definite decrease in enzyme activity, after reacting these inhibitors in whole blood in reaction tubes.
Chapter 1

Introduction

Proteases are a class of enzymes that are essential for the hydrolysis of various peptide bonds in the body. Matrix metalloproteinases (MMPs) in particular are a group of zinc-dependent endopeptidases, which are a subclass of the metzinacin superfamily of proteinases. Abnormal levels of these proteases in blood can be indicative of several diseases, and the ability to rapidly detect and identify them is therefore of immense importance. Also, certain MMPs (MMP-2 and MMP-9) are biomarkers for inflammation in the body, and research suggests that decreasing the levels of these enzymes could increase insulin sensitivity.

Current techniques to measure MMP levels in the blood require initial sample preparation steps, where the blood must be processed before it is tested. This is time-consuming, inaccurate, expensive and difficult to translate into future point-of-care diagnostics (POCs).

The basis for this thesis comes from the research performed at the Heller lab as well as the School of Medicine, both at UCSD. Levels of MMPs and other proteases were detected in whole blood using polyacrylamide gel electrophoresis (PAGE) as the separation technique. PAGE is an electrochemical method by which different biochemical molecules can be separated based on charge, using a constant voltage source. The previous work in the Heller lab involved the use of a synthetically designed, charge changing substrate that was specific to the target enzyme. In the presence of the enzyme being considered, the substrate would get cut into two
fragments – one negatively charged, and one positively charged. The positively charged fragment was linked to a fluorescent tag, thus enabling its detection under a fluorescent detector.

A clinical research trial at the school of medicine involved using the detection of proteases in whole blood as an assay to detect the effect of a low glycemic index diet in overweight volunteers. This trial was assessed over a twelve-week time period. The trial began with the consumption of a liquid meal that had balanced nutrition, followed by a low-calorie dietary regimen for the duration of the study. Using the synthetic substrate, the enzymes were successfully detected in whole blood, making it clear that technique could have wide-spread use as a tool for medical diagnostics.

The effect of certain MMP inhibitors was also analyzed using this whole blood protease assay. Polyphenols present is nature in foods such as grape seeds and apple peel, have shown anti-inflammatory properties by downregulating inflammatory markers such as MMPs. The efficacy of such inhibitors, as well as synthetic inhibitors such as the antibiotic doxycycline, on levels of MMPs was tested. Using the charge-changing substrate, the effect of these inhibitors was first tested in a buffer solution, followed by testing in-vitro in whole blood taken from volunteers which were part of the meal tolerance study.

The synthetic substrate allowed very low limits of detection of the enzymes, and the elimination of sample preparation allows for faster and more accurate results. The detection of MMPs in whole blood, and the effect of their inhibitors in-vitro, can be significant in medical diagnostics, for therapeutic applications, and for future use in devices such as point of care diagnostic devices which are portable and easy to handle.
2.1 Proteases

Enzymes are molecules which catalyze different reactions. Proteases are a class of enzymes that breakdown proteins by hydrolyzing the peptide bond. This class of enzymes can be classified based on the group that is responsible for catalyzing the reaction. Some of these proteases are:

- Serine proteases
- Threonine proteases
- Cysteine proteases
- Aspartate proteases
- Metalloproteases
- Glutamic acid proteases

Of these major classes, metalloproteases are unique in that the water molecule used for hydrolysis forms a complex with the metal ion in the active catalytic center of the protease\(^1\). This metal ion itself is held in position by numerous amino acid residues.
2.1.1 Matrix Metalloproteases

The metzincin superfamily is a subgroup of proteases that has a highly conserved motif. This motif comprises of three histidines that bind to zinc at the catalytic site and a conserved methionine that lies underneath the active site\(^2\). This superfamily of proteases is further divided into four groups: astacins, ADAMs/adamalysins, seralysins, and matrix metalloproteases (MMPs)\(^3\).

MMPs are zinc – dependent hydrolytic enzymes involved in the degradation of the extracellular matrix (ECM) and components of the basement membrane. Apart from degradation, MMP activity is also involved in the release of growth factors in the ECM, which leads to changes in cell-matrix and cell-cell interactions.

MMP activity is controlled at several levels\(^4\). Cytokines such as tumor necrosis factor alpha (TNF-α) as well as certain interleukins such as interleukin-1 (IL-1) and interleukin-6 (IL-6) are responsible for gene regulation\(^5, 6, 7\). MMPs and other proteases are responsible for modulating the activity of these factors and several other pathways in the body which are responsible for homeostasis\(^8\). The enzymatic activity is offset by tissue inhibitors of metalloproteases (TIMPs)\(^9\), which act as natural, endogenous inhibitors of most MMPs\(^10\).

Another level of control of the MMPs lies in the activation of latent zymogens. Zymogens are inactive precursors of enzymes which require some form of posttranslational modification/biochemical change in order to become active enzymes. Plasmin is a powerful activator of most MMPs, promoting cleavage of the latent proenzymes to the active molecule\(^11, 12\). Alternatively, cell-associated
membrane-type MMPs (MT-MMPs) are also involved in activation of metalloproteases by removal of their prodomain\textsuperscript{13}.

Unregulated levels of MMPs can be indicative of various diseases such as hypertension\textsuperscript{14,15}, multiple sclerosis\textsuperscript{16}, physiological shock\textsuperscript{17}, rheumatoid arthritis\textsuperscript{18}, several types of cancers\textsuperscript{19-21}, diabetes\textsuperscript{22-24} and inflammatory diseases such as inflammatory bowel disease\textsuperscript{25}. For this reason, the study of MMPs and their inhibitors is of growing importance in therapeutics and medical diagnostics.

Since the discovery of the first MMP (MMP-1) in 1965\textsuperscript{26}, 23 forms of this class of enzymes (in humans) have been identified. Due to the fact that most MMPs degrade the extracellular matrix, the different types have overlapping cleavage sites and functions. However, they can be roughly classified, based on substrate specificity, into collagenases, gelatinases, matrilysins, membrane type, stromelysins, and unclassified\textsuperscript{27}.

2.1.2 Gelatinases

Gelatinases are a group of MMPs (specifically MMP2 and MMP9), which have a gelatin-binding region in the catalytic domain. MMP-2 (also known as 72 kDa type IV collagenase, gelatinase-A) is produced by osteoblasts and odontoblasts in human hard tissues, as well as in fibroblasts\textsuperscript{28,29} MMP-2 is found in the ECM, bound to type I and type IV collagen. MMP-9 (92 kDa type IV collagenase, gelatinase B) is found in human macrophages and polymorphonuclear leukocytes\textsuperscript{30}. MMP-9 can be expressed by osteoclasts in normal human bone tissues, and it therefore may play a
role in the bone remodeling\textsuperscript{31}. Also, similar to MMP-2, MMP-9 may exist in the ECM bound to type I collagen, laminin or gelatin\textsuperscript{32}.

![Figure 2.1 (a)structure of MMP-2\textsuperscript{33} and (b) Structure of MMP-9\textsuperscript{34}](image)

These enzymes are responsible for the degradation of type IV collagen and gelatin, the main constituents of the basement membrane. They are involved in the proteolysis of the ECM for tissue remodeling, reproduction and embryonic development\textsuperscript{35}. Research shows that gelatinases are involved in the pathophysiology of diseases such as diabetes\textsuperscript{24,36}, hypertension\textsuperscript{15}, acute coronary syndrome\textsuperscript{37}, preeclampsia\textsuperscript{38} as well as breast\textsuperscript{39}, prostate\textsuperscript{40}, colorectal\textsuperscript{41} and bladder cancer\textsuperscript{42}. 
2.2 MMPs in inflammation

Matrix proteolysis is a major component of the inflammatory process, which is associated with many conditions, and MMPs are some of the key enzymes in this process of proteolysis. Therefore, they are considered important constituents in the host response to infectious, traumatic, autoimmune or toxic conditions - all of which fall under the broad category of inflammation.

The inflammatory process is formed by a sequence of cellular responses which depend on incorporating information associated with the following: recognition of the presence of microorganisms or an injury, the buildup and intervention of cells that eliminate invading microorganisms, and the repair of damaged tissues\textsuperscript{43}. In each category there are diseases wherein some members of the MMP family have been upregulated.

There are several approaches to classifying inflammation. Inflammation based on the duration may be classified as follows: per-acute, acute, sub-acute, and chronic. Inflammation is a major component of several diseases\textsuperscript{44–46} including insulin resistance, which is seen in individuals with obesity, type 2 diabetes and cardiovascular disease.
2.2.1 Relating inflammation, obesity and insulin resistance

Research shows that there is a definite correlation between insulin resistance, obesity, cardiovascular diseases (CVDs) and inflammation\textsuperscript{23,48-50}. Two transcription factor signaling pathways have been connected to the proinflammatory effects of insulin resistance: the NF-\textit{kB} pathway, and the c-Jun NH\textsubscript{2}-terminal kinase (JNK) pathway\textsuperscript{51}. Both of these pathways are activated by proinflammatory cytokines such as tumor necrosis factor $\alpha$ (TNF $\alpha$) and interleukins such as interleukin-6. It has been observed that in obese individuals, adipocytes produce large quantities of proinflammatory cytokines such as TNF-$\alpha$ and interleukin-6\textsuperscript{52,53}. Among the several effects produced by TNF-$\alpha$, one is the induction of expression of MMPs such as
MMP-9\textsuperscript{54} and MMP-2\textsuperscript{55}. There are also other theories which suggest the mechanism by which cytokines regulate the activity of gelatinases\textsuperscript{55,56}.

Obesity and insulin resistance can be connected to both localized tissue and systemic inflammation. Inflammatory signaling weakens insulin action, primarily via lipid and cytokine inhibition of insulin receptor substrates and activation of NFκB pro-inflammatory target genes\textsuperscript{45}. NFκB is a transcription factor whose importance has been realized in recent years due to its role in inflammation \textsuperscript{57} and inflammatory diseases\textsuperscript{58}. When activated by pro-inflammatory mediators, NFκB moves to the nucleus where it turns on the transcription of pro-inflammatory genes including IL-1β and TNF-α. The pro-inflammatory signaling pathways get activated via binding of lipid moieties such as lipoxygenase products and saturated fats\textsuperscript{59,60} on the cell surface. Lipooxygenase products can be activated by a high-fat diet, and lead to the increase in omega-6 fatty acids (FAs)\textsuperscript{61}. The omega-6 FAs can eventually be converted into prostaglandins, thromboxanes, leukotrienes, and lipoxins, which are involved in inflammatory signalling.

MMPs have also been found to have an important role in the pathogenesis of several cardiovascular diseases including cardiomyopathy, atherosclerosis, congestive heart failure, restenosis, myocardial infarction (MI), and aortic aneurysm\textsuperscript{62,63}. Aside from cardiomyocyte death, changes in ECM can lead to left ventricular (LV) remodeling after MI\textsuperscript{64}. MMP activity may destabilize atherosclerotic plaques and cause them to rupture, leading to MI. MMP activity is also thought to assist in the transport of vascular smooth muscle cells into the intima of the vessel wall through the
internal elastic lamina. In the intima, these cells multiply and contribute to plaque formation\textsuperscript{65}.

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure2.3.png}
\caption{The correlation between obesity and inflammation}
\end{figure}

2.3 MMPs in other diseases

Another pathological area where MMPs play a key role is in tumor progression. Tumor progression and metastasis require the breakdown of the ECM by release of enzymes such as MMPs to enable invasion of abnormal cells. According to the “three-step” theory of tumor cell invasion, key events in the tumor invasion process include tumor cell adhesion to ECM and its components, ECM degradation
by proteolysis, and finally tumor cell migration into the degraded area\textsuperscript{20}. In certain cases, this invasion of cells is radically affected by the levels of MMPs. TIMPs are endogenously expressed inhibitors of MMPs, and can decrease metastasis of tumor cells. Research indicates that MMP inhibitors could be used for therapeutic purposes to fight tumor progression.

With the presence of MMPs in several pathological conditions, it is therefore of importance to find a technique to identify and quantify these enzymes in a rapid, effective manner.

2.4 Detection:

Earlier, protease activity was generally measured using chromogenic and fluorescence resonant energy transfer (FRET)-based substrates\textsuperscript{66}, zymography\textsuperscript{67}, or fluorescent polarization\textsuperscript{68}. Recently, assays have been developed to detect enzyme activity using bioluminescence resonance energy transfer (BRET)\textsuperscript{69}. Although these techniques can be used for detecting enzymes in plasma or serum, they are not appropriate for detecting protease activity directly in whole blood due to strong background absorption, autofluorescence, and light scattering from other components present in blood\textsuperscript{70}. In order to eradicate these problems, such techniques require sample preparation. The preliminary steps in sample preparation involve centrifugation, filtration, separation of certain components of the blood and addition of anticoagulants which may change the concentration and activity of the enzymes and decrease the efficacy of the assay technique.\textsuperscript{71-73}
For example, certain proteases may get eradicated during the preparation of plasma or serum. Also, calcium is a cofactor necessary for MMP activity, and this cofactor may get sequestered by certain anticoagulants such as sodium citrate. The addition of heparin (another anticoagulant) also has a strong effect on both MMP-9 activity levels in the blood. Furthermore, these steps can cause hemolysis, triggering escape of cytoplasmic proteases into the plasma.

Lefkowitz et al recently demonstrated a simple, efficient electrophoretic technique that uses a charge-changing fluorescent substrate which enables the measurement of protease activity in whole blood. This concept has been used for various proteases such as trypsin, chymotrypsin, elastase, and gelatinases (MMP-2 and MMP-9). Using a simple electrophoretic setup (specifically PAGE; Polyacrylamide gel electrophoresis), proteases can be detected directly in whole blood without any sample preparation. Even at low limits of detection (due to the addition of MMP inhibitors), considerable fluorescent peptide cleavage was seen.

2.4.1 Principle of gel electrophoresis:

Electrophoresis is the passage of electrically charged molecules in solution under the influence of an electric field. Electrically charged molecules move at different speeds depending on the system used and the physical characteristics of the molecules.

There are various types of electrophoresis, one of which is gel electrophoresis. Using a hydrated gel network can be advantageous for electrophoresis in several
ways. For example, as the components of gels are neutral, they remain inactive during electrophoresis and do not interfere with the samples being tested. Also, their mechanical stability allows for post-electrophoretic manipulation, and furthermore, the composition of gels can be changed to vary their porosity for different experiments. One example of a strong gel suitable for separation of both proteins and nucleic acids is formed by polyacrylamide. By cross-linking acrylamide with a methylene bridge, a chemically inactive and mechanically stable gel with controlled porosity can be obtained. PAGE provides a high resolution method for physical-chemical characterization of molecules based on size, conformation, and net charge.

2.5 MMP inhibitors

MMP inhibitors can be classified based on their presence in the body (endogenous and exogenous) or their source (natural or synthetic). The most commonly found endogenous MMP inhibitors are TIMPs.

2.5.1 Inhibitors occurring in nature

These natural compounds include, but are not limited to, long-chain fatty acids, certain polyphenols, and flavonoids. Natural endogenous inhibitors are TIMPs, which can be classified into four major proteins: TIMP-1, TIMP-2, TIMP-3, and TIMP-4. They act by binding to the catalytic domain of MMPs, thereby inactivating
them. In gelatinases, TIMPs create a complex with the proenzyme (zymogene), thus preventing them from transforming to their active form.

TIMPs are found in the heart and in cardiomyocytes, and are composed of a large N-terminal domain responsible for MMP inhibition and a smaller C-terminal domain\(^37\). The transcription of TIMPs is regulated by cytokines and growth factors similar to the ones that control MMP expression, (TNF-a, interleukins, etc.)

Other endogenous inhibitors of MMPs include plasma protein a2-macroglobulin, ovostatins and the reversion-inducing-cysteine-rich protein with kazal motifs inhibitor\(^84\).

2.5.2 Synthetic inhibitors

A typical MMP inhibitor (MMPI) consists of two parts, a zinc(II) binding group and a backbone. The most commonly used synthetic MMPIs are hydroxamic acids. Hydroxamates are monoanionic, bidentate chelators which form hydrogen bonds with several residues within the MMP active site. Although these inhibitors show good in-vitro efficacy, hydroxamic acid MMPIs have not yet successfully completed clinical trials\(^85-87\). However, the hydroxamate moiety is found in certain food and drug administration (FDA) approved drugs such as bufexamac (for skin inflammation treatment), ibuprofam and vorinostat (an anti-cancer drug)\(^88\). Other non-hydroxamic acid MMIs include (based on mode of binding the active site): monodentate ligands, bidentate chelators, and mechanism-based inhibitors\(^27\).
2.5.3 Potential MMP inhibitors:

Research has shown that there are certain exogenous compounds that can act as MMPIs\textsuperscript{89,91,93}, although are not yet through clinical trials. These moieties can be found in nature, or can be synthetically derived drugs which have been traditionally used for treatment of other diseases. In this thesis, synthetic compounds such as doxycycline, and naturally occurring compounds such as polyphenols (found in grape seed extract and apple peel extract) have been studied to test their efficacy as gelatinase inhibitors, as a potential therapeutic for insulin resistance and obesity.

Doxycycline is a commonly used semi-synthetic, broad-spectrum antibiotic\textsuperscript{89}. However, it has also been seen to inhibit inflammation by inhibiting activity of certain MMPs (specifically, MMP-9)\textsuperscript{90}. 

Figure 2.4. Examples of hydroxamic acid based MMPIs
2.5.3.1 Phenols and polyphenols in nature

Proanthocyanidins are a group of polyphenolic bioflavinoids occurring in nature, available in fruits, vegetables, and nuts, and are known to have a broad spectrum of pharmacological and therapeutic effects\(^91\). They show a wide range of biological effects including antibacterial, antialleric, anti-inflammatory, and vasodilatory actions. Grape seeds in particular are rich in procyanidins, a subclass of proanthocyanidins. Grape seed polyphenols are commercially marketed as ‘grape seed extract’ (GSE) in the form of dietary supplements\(^92\). GSE has been shown to be effective as an anti-inflammatory agent, antioxidant\(^93\), and anti-cancer agent.

2.5.3.2 The mechanism of action

Phenolic compounds such as those found in GSE, extra-virgin olive oil, red wine, etc. work together with a variety of molecular targets central to the cell signalling machinery, and can thereby control inflammatory action in a cell\(^58\). The anti-inflammatory activities of polyphenols may include the following mechanism\(^94-97\):

i) pro-inflammatory enzymes such as cyclooxygenase (COX-2) getting inhibited by activation of peroxisome proliferators – activated receptor gamma (PPAR\(\gamma\))

ii) different enzymes and pathways such as tyrosine kinases, phosphoinositide 3-kinase (PI 3-kinase), NF-\(\kappa\)B and c-JUN being inhibited
iii) certain protein kinases including protein kinase C (PKC), mitogen-activated protein kinase (MAPK), serin/threonin protein kinase as well as phase II antioxidant detoxifying enzymes getting activated

iv) modulation of numerous cell survival genes

2.6 Meal tolerance study

The correlation between obesity, inflammation and insulin resistance (and the effect of MMPs therein) is being studied as part of an ongoing clinical research at the school of medicine at UC San Diego. The purpose of this study is to test if a specifically designed diet (consisting of low glycemic index food, fish oil and polyphenols) can improve insulin sensitivity and decrease inflammation in individuals considered to be obese.

2.6.1 Glycemic index.

Glycemic index (GI) is a measure of postprandial blood glucose response to a particular food. It is defined as the area under the glucose response curve after consuming an average amount of carbohydrate from a test food, compared to that of a control food\textsuperscript{98,99}.

The GI of a specific food or meal depends on the nature of the carbohydrate consumed and other factors that affect nutrient digestibility or insulin secretion\textsuperscript{100}. High–GI foods cause an preliminary period of high blood glucose and insulin levels,
followed in several individuals by reactive hypoglycemia, counter-regulatory hormone secretion, and elevated serum free fatty acid concentrations\textsuperscript{101}. GI is hence an important element in the modulation of inflammation, obesity, insulin resistance, energy expenditure, and cardiovascular disease in humans\textsuperscript{102-105}.

2.6.2 Omega-3 Fatty acids (FAs)

Diets composed of excessive \(\omega\)-6 FAs (such as linoleic acid, LA) are pro-inflammatory; \(\omega\)-3 FA-enriched (for example, docosahexaenoic acid, DHA) diets are anti-inflammatory\textsuperscript{106}. An \(\omega\)-3 FA-enriched diet can reverse the detrimental effects of \(\omega\)-6 FA-enriched high-fat diet after a diet switch. \(\omega\)-6 FAs can activate adipogenesis\textsuperscript{107}, and can lead to fat mass accumulation over several generations\textsuperscript{108}.

2.6.3 Body mass index

BMI is the ratio of an individual’s weight, to the square of his/her height; it has the units kg/m\textsuperscript{2}. It is used to classify people into different groups, and is a measure to identify obesity.
Table 2.1 Classification of Overweight and Obesity by BMI

<table>
<thead>
<tr>
<th>General class</th>
<th>Obesity class</th>
<th>BMI (kg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Underweight</td>
<td></td>
<td>&lt; 18.5</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td>18.5 – 24.9</td>
</tr>
<tr>
<td>Overweight</td>
<td></td>
<td>25.0 – 29.9</td>
</tr>
<tr>
<td>Obesity</td>
<td>I</td>
<td>30.0 – 34.9</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>35.0 – 39.9</td>
</tr>
<tr>
<td>Extreme obesity</td>
<td>III</td>
<td>40</td>
</tr>
</tbody>
</table>

Although it should be considered a rough estimate, BMI provides the most holistic measure of obesity as it is the same for both sexes and for all ages of adults.

2.7 Prevalence of obesity and insulin resistance

According to the world health organization (WHO), as of May 2012, obesity is the fifth leading risk for deaths worldwide. Each year, the least possible deaths (in adults) due to overweight or obesity is about 2.8 million.
Figure 2.5. The leading causes of global preventable deaths\textsuperscript{110}. Of these, high blood glucose, high blood pressure (BP), obesity and high cholesterol are all related, and have upregulated markers for inflammation, bringing the total percent of total preventable deaths caused due to inflammation and obesity to about 27%.

Being overweight or obese can cause several deleterious metabolic effects on blood pressure, insulin resistance, triglycerides and cholesterol. An increase in BMI directly correlates with growing risk of CVDs and insulin resistance, as well as increased risk of kidney, colon, prostate, breast, and gall bladder cancer.

There is also an escalation in mortality rates with increasing BMI. The correlation between mortality and diseases can be assessed by disability-adjusted life years (DALYs). DALYs are a measure by which deaths can be measured at different
each DALY corresponds to one year of healthy life that is lost due to a disease or injury.

DALYs are calculated as the addition of years lost due to disability (YLD) and years of life lost due to premature mortality (YLL). It has been seen that obesity and high blood glucose are among the top ten DALYs worldwide, making them some of the leading causes of attributable global mortality.

Low fruit and vegetable intake, high blood glucose, high BMI, lack of exercise, high cholesterol, high blood pressure, as well as alcohol and tobacco use are risk factors responsible for more than 50% deaths due to CVDs worldwide, which are the number one cause of global deaths each year.

The growing number of deaths each year due to obesity and the complications that arise from it, emphasises the need for a way to reduce the population having a high BMI. Thus, it is very important to find a diet that promotes a fit lifestyle and leads to a healthy body weight. It is also important to be able to assess the effects of such a diet at a molecular level, which is what this thesis aims to do.

As mentioned before, the Meal Tolerance test in UCSD aims to link obesity, inflammation and insulin resistance; and how low GI foods can not only help reduce obesity, but also decrease inflammation. As part of this study, MMP2/9 activity was used as a marker for inflammation. The technique developed by Lefkowitz et al. was used to measure MMP2/9 activity directly in blood from patients. Moreover, different MMP2/9 inhibitors were tested ex-vivo against the enzyme, in an aim to test their
effects on the enzyme activity; and thus suggesting ways of reducing protease activity, and therefore inflammation in the blood of patients.
Chapter 3

Experimental Section

3.1 Material

The experimental section of this study has primarily three parts: preparation of the substrate, reacting the substrate with sample (followed by electrophoresis), and analysis of the sample after electrophoresis.

The material used for this study consisted primarily of the gel electrophoresis unit, a transilluminator, and a scanning system, along with several reagents and blood taken from volunteers as part of the meal tolerance clinical study.

3.1.1 Gel electrophoresis:

Gel electrophoresis works on the principle of charge separation. On application of an electric field, components of a system move with opposing charge move away from each other. This principle is based on the governing equation for steady state velocity of charged particles in an electric field\(^{112}\), given by

\[ v_{ss} = \frac{E \cdot q}{f} \]

Where,

- \( E \) = electric field strength,
- \( f \) = frictional coefficient,
- \( q \) = net charge on the molecule
The frictional coefficient (f) can be defined as the frictional resistance to movement. It is influenced by several factors including the mass of the molecule, buffer viscosity, and porosity of the gel matrix.

In gel electrophoresis, an electric field can be established by applying a constant voltage ‘V’ to a pair of electrodes separated by distance ‘d’. In this case, the electric field strength is now,

\[ E = \frac{V}{d} \]

Due to this concept of charge separation, the samples used in this thesis study (blood added to a substrate with a fluorescent tag) show a band of fluorescence migrating towards the anode, as this cleaved portion of the substrate is positively charged, and the anode is negatively charged. This band is completely separated from the background fluorescence, which moves towards the cathode.
3.1.2 Storm scanner

The scanning system used to analyze gels, following gel electrophoresis, was Storm 860 Molecular Imager Gel and Blot Imaging System.

This imaging system is used for autoradiography, direct fluorescence for nucleic acid and protein gel analysis, and chemifluorescence for rapid blot analysis. It uses reusable storage phosphor screens to deliver filmless high-resolution imaging and precise quantification. Storm exhibits a linear response to fluorescent signal intensities and these intensities are then measured using ImageQuant. ImageQuant is automated software used for the analysis of electrophoresis gel images.
Following image acquisition using the Storm Scanner and ImageQuant, the scanned images were analyzed using ImageJ, a Java-based image processing system.

3.1.3 Transluminator:

The transluminator consists of a fluorescent imager attached to a high resolution camera, through which the images are captured.

3.2 Substrate action

3.2.1 Mechanism of action of substrate on proteases

The synthetically derived substrate consists of a chain of amino acids, with the entire peptide sequence having an overall negative charge. When reacted with sample solution, if the desired protease is present (in this case, MMP-2/9) in the sample, it will cleave the substrate at a specific site. The substrate is divided into two fragments, one of which is negatively charged, and the other is positively charged. This positively charged fragment is tagged with a fluorophore (Bodipy-FL).
Table 3.1 sequence of whole and fragmented substrate

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole</td>
<td>acetyl-N–GDPVGLTAGAGK-(ε-BodipyFL)-NH₂</td>
<td>-1</td>
</tr>
<tr>
<td></td>
<td><strong>Fragments</strong></td>
<td></td>
</tr>
<tr>
<td>C-terminal</td>
<td>Ac-N-GDPVGL-O</td>
<td>-2</td>
</tr>
<tr>
<td>N-terminal</td>
<td>H₃N-TAGAGK--NH₂</td>
<td>+1</td>
</tr>
</tbody>
</table>

3.2.2 Principle of action of substrate in gel electrophoresis

After a specific amount of time, an aliquot of this solution mixture is dispensed into a vertical PAGE setup, and electrophoresed for 10 minutes at a constant voltage. The gel electrophoresis unit is set up such that the positively charged components of the sample move through the gel towards the bottom, whereas the negatively charged portion remains at the top. In this manner, the presence of certain proteases is detected. Also, the intensity of fluorescence is directly proportional to the amount of enzyme present, and thus the fluorescence signal is quantified to indicate the amount of protease present.
3.3 Substrate synthesis:

This synthesis was done by Aapptec (Louisville, KY), and Bodipy FL-SE (Invitrogen, Carlsbad, CA, USA) was then labeled onto the lysine residue’s amine group. The labeling was done by reacting equal volumes of the substrate (10mg/mL) in 100mM NaHCO₃ (pH 8.2) and the fluorophore (also 10mg/mL) in DMSO for 1 hour. The labeled product consists of the following sequence: acetyl-N–GDPVGLTAGAGK-(e-amino-BodipyFL)-NH₂. Recombinant human MMP-2 (M9070) and recombinant human MMP-9 (M8945) obtained from Sigma-Aldrich (St.
Novex pre-cast 1-mm thick 20%T 2.6%C Polyacrylamide TBE gels were obtained from Invitrogen.

The starting concentration of the gelatinase-specific substrate was 5mg/mL. The stock solution of the substrate was then diluted in 1x PBS (pH 7.8) to achieve a final concentration of 0.45mg/mL.

3.4 Detection of MMP in 1X PBS:

After synthesis, conjugation and dilution of the substrate to 0.45 mg/mL, it was tested with recombinant human MMP-2, and recombinant human MMP-9. An initial stock solution of 1μM of each of the proteases was prepared, and then further diluted in phosphate buffer saline (PBS) to give a concentration gradient profile.

With a specific molarity and given weight of the gelatinase, the volume was measured using the following formula:

\[
vol (L) = \text{weight (g)} \times \frac{1}{MW} \times \frac{mol}{g} \times \frac{1}{M mol} \]

Using 10 ug of the gelatinase with a desired concentration of 1μM, the stock solution was prepared, and further diluted to give the different concentrations of the enzyme. (The molecular weight of MMP-2 is 92 kDa or 92 * 10^3 g/mol, and the molecular weight of MMP-9 is 92 kDa). Seven varying volumes of the protease were dispensed into respective gelatinase-specific substrate aliquots to achieve the
following enzyme concentrations: 1000, 800, 500, 100, 50, 10, and 5 nM. 1x PBS (pH 7.8) was added to the ninth substrate aliquot to serve as a negative control.

These enzymes were then reacted with the substrate for 30 minutes (at room temperature), with the total reaction volume being 7.7 uL. After 30 minutes, 6 µL aliquots were dispensed directly into the lanes of Novex Polyacrylamide gels submerged in 0.5× TBE (44.5 mM Tris-Borate, 1 mM EDTA, pH 8.0) and electrophoresed at 500 V for 10 min. Following electrophoresis, the gels were imaged on a BioDoc-It System with a Model M-26 transilluminator (UVP, Upland, CA) with wavelengths of 302 nm for excitation and 550-580 nm for emission. Using a Storm 840 gel scanner, which has a 1000 V photomultiplier tube with a 450 nm excitation filter and a 520 nm long-pass emission filter, these gels were imaged (Storm scanner obtained from Molecular Dynamics, Sunnyvale, CA). The settings of the scanner set to: fluorescence mode, high sensitivity, 100 µmpixels. The fluorescent signal from these images was then quantified using ImageJ open-source software, and the background fluorescence was removed by subtracting it from the negative control.
For the same molarity, it was seen that the signal for MMP-9 was much stronger than that for MMP-2. In subsequent experiments, MMP-9 was used as the enzyme of choice for calibration studies with MMPIs.

3.5 Detection of MMP inhibitors in 1X PBS

3.5.1 Detection of GSE in 1X PBS with MMP-9

Polyphenols such as those found in GSE, inhibit the activity of gelatinases. In this study, the extent of inhibition was analyzed using recombinant human MMP-9, along with the charge-changing substrate.
The limit of detection of this inhibitor was confirmed by measuring the activity of known concentrations of GSE in the presence of a fixed amount of MMP-9 enzyme in a PBS buffer system. Research done in the past by Ray et al.\textsuperscript{113} showed that a dose of 100 mg per kg per day of GSE was considered a safe dosage in rodents, when studied over a twelve-month period. In this study, to measure the efficacy of the extract, different concentrations were considered in incremental amounts, ranging from 0 to 1000 mg/L. These volumes of GSE were reacted with a fixed volume of MMP-9 (500nM) and with the substrate solution (0.45 mg/mL). The final GSE concentrations were 1000, 500, 250, 100, 50, 10, 5, and 0 nM. After a 30 min incubation period at room temperature, the 6μL aliquots were dispensed into the gels, electrophoresed, visualized, and quantified as described in the previous section.
Figure 3.3: Calibration of MMP-9 activity in 1X PBS with GSE

(a) Image of scanned gel showing activity of MMP-9 in buffer, (b) table of different GSE concentrations, corresponding to different lanes of gel in above image; (c) graph of MMP-9 activity in 1X PBS, when mixed with different GSE concentrations

It was seen that there was a definite effect of the GSE on fluorescent signal intensity, and therefore, a significant effect on the levels of MMP-9. For experiments using GSE mixed with blood, a fixed volume was considered, which showed definite inhibition, but was within the limit for daily dietary consumption.

3.5.2 Detection of Doxycycline in 1X PBS with MMP-9

Doxycycline, a widely used broad-spectrum antibiotic, has also been known to show MMP inhibitory activity. Using the same procedure as that used for GSE, the
effect of this drug on MMP-9 was tested. An average doxycycline dose is 100 mg, twice daily. After calculating against average blood volume, this concentration is about 0.04 mg/mL; therefore the range of Doxycycline tested was 0 to 4 mg/L. The volumes added to the reaction tubes varied until the final concentration of the drug in the tubes was as follows (in mg/mL): 4, 1, 0.5, 0.1, 0.04, 0.01. PBS was added to the tubes such that all reaction tubes would have the same total volume. The substrate solution (0.45mg/mL) was reacted with a fixed volume of MMP-9 (200 nM), and the total sample volume was 8.4 uL. 6μL of each sample was then loaded onto the gel, electrophoresed, visualized, and quantified as described in section 3.4.

Figure 3.4: calibration of MMP-9 activity in 1XPBS with doxycycline
(a) Image of scanned gel showing activity of MMP-9 mixed with doxycycline in buffer, (b) table of different doxycycline concentrations, corresponding to different lanes of gel in above image; (c) graph of MMP-9 activity in 1X PBS, when mixed with different doxycycline concentrations
Doxycycline showed a significant inhibition of MMP-9 activity, even at concentrations as low as 0.01 mg/mL (figure 3.4). Using the information from the spiked calibration curves for MMP activity, a fixed volume of the inhibitors was tested in whole blood from volunteers that were insulin resistant.

3.6 Meal tolerance research

The meal tolerance study is an ongoing clinical trial, done by Drs. Sears and Herbst at the UCSD School of Medicine. Forty volunteers, consisting of both men and women between the ages of 21 and 55 with a BMI ranging from 30 to 39 kg/m², have been selected based on initial screening process. The trial consists of a twelve-week study to detect changes in inflammation and insulin sensitivity after consistently following a diet with a low glycemic index, enriched with omega-3 FAs.

In this study, the volunteers have been asked to eat a diet of only 1500 calories a day for a twelve-week period, in the form of nutrient shake mixes, bread-type products, oil capsules and powder capsules. The study is randomized - half the volunteers consume the test diet and the other half consume a placebo control diet. The placebo diet contains high glycemic index foods, corn oil capsules, and corn starch capsules. Blood is collected from the patients on the first and last day of the study, and tested for levels of MMPs using the charge-changing fluorescent substrate. The volunteers are given a nutrient shake on the day of blood collection, immediately after the first time point for collecting blood. This nutrient shake (Ensure Plus®) is a
high-calorie shake, that is also rich in proteins and several essential vitamins and minerals, as given in table 3.2

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Amount per serving</th>
<th>% Daily Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat</td>
<td>11 g</td>
<td>17</td>
</tr>
<tr>
<td>Protein</td>
<td>13 g</td>
<td>26</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>50 g</td>
<td>17</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>10 mg</td>
<td>3</td>
</tr>
<tr>
<td>Sodium</td>
<td>220 mg</td>
<td>9</td>
</tr>
<tr>
<td>Potassium</td>
<td>400 mg</td>
<td>11</td>
</tr>
</tbody>
</table>

A portion of the meal tolerance study was done as a part of this thesis. Blood collected from patients (on the first or last day of the study) was immediately tested for presence of gelatinases. At different time points after consuming an EnsurePlus nutrient shake, with time = 0 being the time point right before consumption, a small volume of blood was drawn from the volunteers. Other time points at which blood was collected were t = 30, 60, 90, and 120 minutes after consumption of the shake.

A small sample of blood (3.5 uL) was then mixed with the gelatinase-specific fluorescent substrate, and incubated at room temperature for 30 minutes. After this period, 6μL aliquots of each sample were then loaded onto the gel, electrophoresed, visualized, and quantified as described in section 3.4.
This experiment was repeated for over eight volunteers (both men and women) and was pooled together to get the holistic, average results. These results were compared to the average results obtained from blood taken from normal volunteers (n=2). Normal volunteers were considered those that were not insulin resistant, had normal blood glucose levels, and maintained a fit, healthy lifestyle.

Graph 3.2: average results of meal tolerance from insulin resistant and healthy volunteers

From the average results pooled together from several volunteers, it was seen that there wasn’t a significant change in MMP activity over time between normal and insulin resistant volunteers. As this is an ongoing trial with a total of forty insulin-resistant volunteers, this trend may change.
Another parameter used to check the response between insulin resistant and normal volunteers was done by comparing the percent change in MMP activity, using time = 0 min as the baseline time point.

Graph 3.3: percentage change in activity, compared to the time = 0 min

It was seen that there was a definite difference in the trend of MMP activity after consumption of the meal. In the healthy, control subjects, the MMP levels seemed to increase, reach a peak, and then decrease with time. However, in the insulin resistant volunteers, the MMP levels seemed to progressively decrease with time.

The effect of this study was also compared for two days of the study: day 0 (beginning of study) and day 84 (end of study). The MMP activity at different time points on these days was evaluated – these time points were t = 0, 30, 60, 90 and 120 minutes after consumption of Ensure Plus® shake.
Graph 3.4: comparison of MMP levels on first and last day of study (n = 4)

There was an overall decrease in MMP activity over the twelve-week period, indicating that this meal study was improving inflammation conditions in the volunteers.

3.7 Meal tolerance study with MMPIs

3.7.1 Meal tolerance with doxycycline

The effect of MMPIs was tested on whole blood taken from volunteers which were part of the meal tolerance study. A fixed volume of Doxycycline stock solution was added to the reaction tube such that the final concentration of the drug in each
tube was 0.04 mg/ml. Whole blood from volunteers was collected at t = 0, 30, 60, 90, and 120 minutes after consuming the nutrient shake. The blood was then added to each tube and it was incubated at 37 C for 30 minutes. After 30 minutes, the substrate was added, and the tubes were incubated at room temperature and allowed to react for another 30 minutes. After this 60 minute total reaction time, 6μL aliquots of each sample were then loaded onto the gel, electrophoresed, visualized, and quantified as described in section 3.4

Graph 3.5: Average meal tolerance with Doxycycline (n = 2)

Graph 3.5 shows significant effect of doxycycline on the level of MMP activity.
3.7.2 Meal tolerance with GSE

The inhibitory effect of GSE on MMPs in whole blood was also tested. A standard stock solution of GSE was prepared (1000 mg/L), and a fixed volume was added to each reaction tube such that the final concentration of GSE in each tube, after adding the blood and substrate, was 0.4 mg/L. Blood was added to the reaction tubes, and allowed to react with GSE for 30 minutes at 37°C. The substrate was then added to each tube, and incubated at room temperature for 30 minutes, after which 6 μL aliquots from each sample were loaded into the gel and electrophoresed. After electrophoresis, the gels were imaged using the transluminator and scanned using the Storm scanner, as described in section 3.4

The average results pooled together from three volunteers, to whose blood GSE had been added in-vitro, was charted on a graph to assess the effect of this polyphenolic compound.
From graph 3.6, it can be seen that there is a definite change in MMP activity after addition of the GSE inhibitor.

3.8 Statistical testing

For determining the changes between groups, analysis of variance (ANOVA) testing was carried out. As there were two major testing criteria (first and last week of study, insulin resistant and normal volunteers), and each criteria had several individuals, a two-way ANOVA with multiple repeats was considered.
Graph 3.7 Two-way ANOVA testing of insulin resistant versus control volunteers

As seen from graph 3.7, there is a large variation in the different individuals at each time point. This may be due to differences in weight, age, gender, and so on. Further testing will have to be carried out to determine the cause for such large differences in values.

Two-way ANOVA was also carried out for determining the variance between individuals on the first and last day of the study.
Graph 3.8 Two way ANOVA comparing week 0 versus week 12 of the study

It was seen that with one exception, all individuals fell within a certain range on the Y-axis ($2 \times 10^6$ to $5 \times 10^6$), indicating that the results are quite statistically significant.
Chapter 4:

Results and future direction

This study has shown that measuring the levels of proteases such as MMPs in whole, unprocessed blood in possible in an efficient, accurate manner using the charge-changing fluorescent substrate. The amount of fluorescence directly corresponds to the amount of substrate cleaved, which in turn signifies the amount of enzyme present in the blood sample.

When the MMP inhibitors were used in a 1XPBS buffer solution, there was a significant change in activity seen in the samples, depending on the concentration used.

4.1 Meal tolerance result

As mentioned before in chapter 3, the aim of the meal tolerance study was to check the effect of a certain diet on obese individuals, and ascertain if this diet could decrease inflammation and increase insulin resistance in the body.
4.1.1 Meal tolerance in obese versus normal volunteers

In this part, the expected results were an increase in MMP activity after consuming the liquid shake, and higher MMP values in insulin resistant volunteers compared to the normal. However, the experiments showed that MMP values only increase initially in the healthy volunteers, and progressively decreased in the insulin resistant ones.

One hypothesis for this involves the role of TIMPs in chronic inflammation. As obese individuals, the insulin resistant volunteers have persistent chronic inflammation in their bodies, and due to this, they may have higher levels of TIMPs in their bodies. Thus, after consuming a meal, MMP values may not increase significantly because TIMPs are preventing their activation. Further tests (such as Western blot, or gel electrophoresis with patients’ blood spiked with MMP) will have to be carried out to evaluate the reason behind this observed trend, which differs from the expected result.
4.1.2 Effect of diet on MMP levels

Blood was collected from the volunteers on the first and last day of the twelve-week study, and results expected were a decrease in the levels of MMPs after this time period. However, the experiments showed that there was only a slight decrease in MMP levels over the 12-week period, although all the volunteers did lose weight over that time. This may be due to substrate specificity with respect to different MMPs. As seen in graph 3.1, the synthetic substrate showed a higher affinity for MMP-9 than it did for MMP-2. Some changes in the charge-changing substrate may lead to a fluorescent substrate with higher specificity for gelatinases, and therefore, more accurate data. Also, some of these volunteers were on a placebo diet, and they may not have shown a very significant change in their MMP levels. Further tests with a separation of the placebos from the test subjects may show a difference in data.

Another important factor to consider is the experimental conditions. All of the experiments in this study were carried out in a laboratory, within hours of collecting the blood from the volunteers. The results of this test will be different when done in vivo, and certain other parameters such as presence of other enzymes, various reactions in the body, etc. should be considered while evaluating the results from these experiments.

In conclusion, this meal tolerance study may show a decrease in MMP levels over time, once all forty volunteers have completed the study, after adhering to all the
required rules. Further experiments can be carried out to increase the specificity of substrate, as well as to see the effect of this diet on both placebo and test volunteers (when considered separately).
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109. adapted from (“Clinical guidelines on the identification, evaluation, and treatment of overweight and obesity in adults.”, WHO 1998)


