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A whole blood protease assay

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A Whole Blood Protease Assay

A Dissertation submitted in partial satisfaction of the requirements
for the degree Doctor of Philosophy

in

Bioengineering

by

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2010

The Dissertation of Roy Brian Lefkowitz is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2010

DEDICATION

To my mother, my late father, my brother, and my wife,

Thank you very much for all of your love and support.

I owe my success to all of you.

EPIGRAPH

“I've failed over and over and over again in my life and that is why I succeed.”
Michael Jordan

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LIST OF ABBREVIATIONS

Ac ..... acetyl

ADAM ..... a disintegrin and metalloprotease

APS ..... ammonium persulfate

BFL ..... Bodipy FL

CLIA ..... clinical laboratory improvement amendments

DMSO ..... dimethylsulfoxide

EDTA ..... ethylenediaminetetraacetic acid

ε ..... epsilon amino

FRET ..... fluorescence resonant energy transfer

FG ..... focusing gel

HMW ..... high molecular weight

KLK ..... kallikrein

LOD ..... limit-of-detection

MODS ..... multi-organ dysfunction syndrome

MOF ..... multi-organ failure

MMP ..... matrix metalloprotease/metalloproteinase

OGTT ..... oral glucose tolerance test

PBS ..... phosphate buffered saline

PG ..... poly-L-glutamic acid

PG1 ..... poly-L-glutamic acid 1

PG2 ..... poly-L-glutamic acid 2

POC ..... point-of-care

PSA ..... prostate specific antigen

RFU ..... relative fluorescence unit

TEMED ..... tetramethylethylenediamine

TBE.....tris borate EDTA

TIMP..... tissue inhibitor of metalloproteinases

TPCK.....N-tosyl-L-phenylalanyl chloromethyl ketone

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ABSTRACTS

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ABSTRACT OF THE DISSERTATION

A Whole Blood Protease Assay

by

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Doctor of Philosophy in Bioengineering
University of California, San Diego, 2010
Professor Michael J. Heller, Chair

The measurement of protease activity in blood is important for the development of novel diagnostics and for biomedical research. Presently, most protease assays require a considerable amount of sample preparation, making them time-consuming, costly and less accurate. Sample preparation also increases the complexity of these assays, precluding them from point-of-care applications.

The current dissertation explores the development of a simple and rapid electrophoretic assay for measurement of protease activity directly in microliters of whole blood. This assay utilizes charge-changing fluorescent peptide substrates that produce a positively charged fluorescent product fragment upon cleavage by the target

enzyme. This fragment is then rapidly separated from whole blood by electrophoresis and quantified with a fluorescent detector. Charge-changing substrates were developed for detection of α-chymotrypsin, trypsin, elastase, matrix metalloproteinase-2 and matrix metalloproteinase-9. Kinetics and specificity studies were then performed in order to make comparisons with substrates from previous assays. Electrophoretic conditions were optimized in order to achieve lower detection limits. During this optimization, various electrophoretic gel formats were explored, such as agarose and polyacrylamide slab gels. A novel technique, called polyanion focusing gel electrophoresis, was developed in order to achieve even lower detection limits. In this method, we used to polyanionic poly-L-glutamic acid-doped polyacrylamide gels to focus the fluorescent cleavage product and markedly improve the detection limits of the assay toward clinically relevant levels of protease activity.

For all of this study's five target proteases, this technique was able to demonstrate detection limits that were below the normal reference levels established in the literature. Using this assay, protease activity was measured in blood, plasma, and serum samples from rats, mice, or humans that have type II diabetes, pancreatic cancer, or that are undergoing physiological shock. In these small proof-of-concept studies, the assay has been able to consistently measure both normal baseline and abnormal levels, showing that this technology may be useful for the development of novel diagnostics. This straightforward technique now allows for the rapid measurement of clinically relevant levels of protease activity in microliter volumes of whole blood, providing a useful tool for the development of novel point-of-care diagnostics.

Chapter 1:

Introduction to Protease Detection

1.1 Motivation for Protease Detection

Proteases (a.k.a. proteinases, peptidases, or proteolytic enzymes) are a class of enzymes that catalyze the breakdown of proteins through the hydrolysis of specific peptide bonds [1]. Crucial to every aspect of the life and death of an organism, these enzymes catalyze a wide range of important physiological reactions. For example, they are involved in the digestion of food, coagulation, fibrinolysis, apoptosis, angiogenesis, and in the complement system. Analysis of over a hundred complete genomes has shown proteases comprise about 2% of all gene products. There are well over a 1000 known proteases. Proteases are a part of a larger class of enzymes known as hydrolases, which catalyze the hydrolysis of a chemical bond. They can be subdivided into two main groups, depending on whether they cleave peptide bonds of terminal (exopeptidases) or nonterminal (endopeptidases) amino acids. The latter group can be further subdivided into four major categories, based on their catalytic mechanism (e.g. aspartic proteases, cysteine proteases, metalloproteases, and threonine proteases).

Proteases play an important role in the initiation and progression of many major diseases and medical conditions including cancer [2-16], physiological shock [17, 18], diabetes [19-23], hypertension [24, 25], acute coronary syndrome [26], pancreatitis [8, 27, 28], preeclampsia [29-31], and inflammatory bowel disease [32, 33]. As a result,

many proteases have been examined for their utility as potential diagnostic biomarkers and as therapeutic drug targets (e.g. inhibiting retropepsin in the treatment of AIDS [1]). In general, previous enzyme detection techniques do not meet the performance parameters needed for rapid and sensitive detection of protease activity in whole blood because they all require sample preparation. These processing steps make protease assays more costly, time consuming, and considerably less accurate, limiting their diagnostic utility. Sample preparation also precludes these assays from point-of-care (POC) applications. The subject of this dissertation is the development of a novel assay that overcomes this major limitation, eliminating the need for sample preparation and allowing the direct measurement of protease activity in whole blood.

This novel assay opens up new doors in biomedical research and in diagnostic development. It provides a new tool for furthering the understanding of disease mechanisms by allowing, for the first time, the measurement of protease activity directly in unprocessed samples. It also provides a new tool for biomarker and drug target discovery as this technology facilitates the ability to perform rapid and accurate multiplexed screening of protease activity in clinical samples. Furthermore, upon validation of a potential protease biomarker or drug target, this assay can be used for therapeutic drug monitoring and for the development of novel POC diagnostics.

In the first chapter, evidence is presented to show the role of proteases in many major diseases and medical conditions, ranging from physiological shock to cancer. This provides motivation for measuring protease activity in whole blood both for biomedical research and in the development of novel point-of-care diagnostics. This is followed by a

review of current protease assays, emphasizing that these assays all suffer from the requirement of sample preparation. Next, the numerous issues with sample preparation are discussed, providing the rationale that sample preparation significantly reduces the accuracy of prior protease assays and precludes them from POC applications. The first chapter closes with a brief discussion detailing how charge-changing substrates overcome this fundamental limitation.

The second chapter discusses the initial development of a whole blood protease assay, using fluorescence-labeled charge-changing peptide substrates. In this chapter, two substrates are developed: (1) a common substrate for both α-chymotrypsin and trypsin and (2) a trypsin-specific substrate. The third chapter discusses the development of a new electrophoretic technique, polyanionic focusing gel electrophoresis, which concentrates the fluorescent signal of the charge-changing substrate assay in order to achieve lower, *clinically relevant* detection limits. The fourth chapter is an extension of the developments of the second and third chapter, presenting the design and characterization of four new substrates for the whole blood detection of elastase, α-chymotrypsin, matrix metalloprotease-2 (MMP-2), and matrix metalloprotease-9 (MMP-9). The final chapter discusses the application of the charge-changing substrate assay toward the detection of protease activity in plasma and in whole blood in physiological shock, diabetes, and in pancreatic cancer.

1.2 Proteases in Physiological Shock

A discussion of the role of proteases in disease must begin with physiological shock since this was the initial motivating application for the development of a whole blood protease assay. In recent years, pancreatic proteases have been implicated in the progression of physiological shock. Physiological shock (a.k.a. circulatory shock) is a serious life-threatening medical condition where the cardiovascular system fails to perfuse tissues adequately, resulting in a widespread impairment of cellular metabolism and eventually progressing, in a matter of hours, to multi-organ dysfunction syndrome (MODS) (a.k.a. multi-organ failure, MOF) and death [34]. It has the second highest hospital mortality rate at 51.5% in 2005 and is a major killer in hospitals worldwide [35]. Clinical manifestations, often occurring in later stages, include: hypotension; a rapid, weak pulse; moist, clammy skin; profuse sweating; chest pain; unconsciousness; feeling weak or nauseous; hyperventilation; feeling anxious, agitated, or confused; dizziness, faintness, or light-headedness; and blue lips and fingernails [34].

Physiological shock, in general, is currently diagnosed symptomatically in late stages. There are currently no biomarkers for diagnosing the early stages of most forms of shock. In the specific case of sepsis and septic shock, there are more then 80 biomarkers developed, such as C-reactive protein and procalcitonin, which are predictive of morbidity and mortality [36]. However, lack of availability, long turnaround times, and nonstandardized assays and cutoff values have limited their practical use. In more recent studies, increasing evidence shows an association between physiological shock and an inflammatory cascade [37, 38]. The inflammatory cascade is accompanied by

activation of cells, expression of pro-inflammatory genes, down regulation of anti-inflammatory genes, attachment of leukocytes to the endothelium, elevated permeability, thrombosis, mast cell degranulation, apoptosis, growth factor release, and many other events [39]. Recent research on trigger mechanisms of the inflammatory cascade in shock and MODS showed that there exists an enhanced level of proteolytic enzymes in blood that are targeted towards a variety of autologous protein and lipid structures [18, 37, 40, 41]. This enzyme activity is not blocked to the same level as in non-disease control blood samples.

In shock, digestive enzymes (e.g. chymotrypsin, trypsin, elastase) synthesized in the pancreas as part of normal digestion find entry into blood via the wall of the intestine (Figure 1.1) [37]. The injuries or insults to the body that trigger physiological shock are often associated with reduced blood flow in the intestine, which in turn triggers an increased epithelial and endothelial permeability. The elevated permeability allows pancreatic enzymes to enter the systemic circulation via the portal vein, the intestinal lymphatics, and via the peritoneal cavity [42]. The digestive enzymes have the ability to degrade almost all biological tissues and molecules and produce inflammatory mediators (toxic protein and lipid fragments). Proteases cause “auto-digestion” of matrix proteins and cell dysfunction, self-digestion of tissues, multi-organ failure, and eventually death. Thus, the detection of these proteases in the blood thus has the exciting potential to diagnose the early stages of physiological shock.

1.3 Proteases in Other Diseases

Abnormal levels of proteases and other types of degradative enzymes (e.g. amylases and lipases) have been observed in the serum or plasma of patients with pancreatitis [8, 27, 28], inflammatory bowel disease (IBD) [32, 33], hypertension [24, 25], acute coronary syndrome [26], diabetes [19-23], preeclampsia [29-31] and in many types of cancer (including pancreatic) [2-16]. In a study conducted by Ventrucci et al., the serum levels of amylase, pancreatic (P) isoamylase, lipase, trypsinogen, and elastase 1 were measured in patients with acute pancreatitis, chronic pancreatitis, pancreatic cancer, and in patients with nonpancreatic abdominal pain [8]. The individual enzyme levels were then each analyzed for their diagnostic utility. For acute pancreatitis, the results of this study showed that there are abnormally elevated levels of all of those enzymes within the first 48 hours of onset, with more persistent elevation due to elastase 1, lipase, and trypsinogen. Using their best diagnostic cut-off value of 700 ng/dL for the most persistently elevated enzyme, elastase 1, they could achieve 100% sensitivity and 98.5% specificity using elastase 1. For patients with chronic pancreatitis, and only in patients with a painful relapse or with pancreatic cysts, elastase 1 was the most frequently elevated enzyme, in 79% and 80% of those cases, respectively. In pancreatic cancer, there were elevated levels for each of these enzymes in many of the patients, with the most frequent abnormal elevations due to elastase 1 (in 35% of patients), but none of the individual enzymes had sufficient sensitivity or specificity to be clinically useful for diagnosis. This study, however, only explored the sensitivity and specificity of diagnosis of these diseases based on the levels of a single biomarker; it is still left to be seen if

simultaneous measurement of several protease biomarkers can provide sufficient sensitivity and specificity for diagnosis of pancreatic cancer.

In another study by Heikius, serum P-isoamylase, amylase, and lipase levels were measured from patients with IBD [32]. In this study, there were abnormally elevated levels of amylase and lipase in 11% and 7%, respectively, of the total study group. The corresponding prevalences in patients with Chron's disease were 17% and 9%, those in ulcerative colitis 9% and 7%, and those in indeterminate colitis 10% and 5%, respectively. High levels of serum amylase and pancreatic isoamylase were associated with extensive colonic disease ($p<0.005$) and high levels of amylase, but not lipase, were significantly elevated in patients with primary scloresing cholangitis. Thus, pancreatic enzymes are elevated in a significant proportion of patients with IBD, and the enzyme increase was associated with a more extensive and active disease.

Further studies by DeLano et al. showed that matrix metalloproteases play a role in the pathophysiological mechanism behind cell membrane receptor cleavage in arterial hypertension [24]. In spontaneously hypertensive rats (SHR), immunohistochemistry and in vivo microzymography revealed elevated levels of matrix metalloprotease-1 (MMP-1) and -9 (MMP-9) activity as well as a corresponding increase in insulin receptor cleavage. Elevated protease activity was also observed in SHR plasma using a fluorogenic protease assay (EnzCheck, Molecular Probes). Together, this evidence shows that elevated MMP activity leads to proteolytic cleavage of the insulin receptor and the subsequent development of insulin resistance seen in hypertension.

Using a sandwich enzyme immunoassay, Kai et al. showed elevated peripheral blood levels of MMPs in patients with acute coronary syndromes (ACS) [26]. On the day

of admission to the hospital (day 0), serum MMP-2 in patients with unstable angina (UA) and acute myocardial infarction (AMI) was two times greater then in control subjects. Plasma MMP-9 in patients with UA and AMI on day 0 was elevated by threefold and twofold versus that in control subjects, respectively.

Using enzyme-linked immunosorbent assay (ELISA) to measure MMP-2 and -9 levels in plasma derived from peripheral blood from patients with type 1 diabetes and from a control group, Maxwell et al. observed significantly elevated levels ($P < 0.015$) of MMP-9 and no significant elevation of MMP-2 [20]. Using the same methods, Lee et al. observed significantly elevated MMP-2 ($P < 0.05$) and no significant elevation of MMP-9 in type 2 diabetes [19].

MMPs have been implicated in the process of tumor growth, invasion, and metastasis [5]. Numerous studies in a variety of tumor types, including lung, colon, breast, and pancreatic carcinomas, have shown an overexpression of MMPs in malignant tissues in comparison to adjacent normal tissues. They have been associated with an aggressive malignant phenotype and adverse prognosis in patients with cancer. Zucker et al. explored the possibility that some of these MMPs synthesized in the tissues would seep into the bloodstream [10]. Toward proving their hypothesis, they used ELISA to measure MMP levels in patients with colorectal, breast, prostate, and bladder cancer. They found, in all of those forms of cancer, that most patients with aggressive disease had elevated plasma levels of MMP-9. Furthermore, they found that high levels of MMP-9 in patients with advanced colorectal cancer were associated with shortened survival. On a side note, Zucker et al. also found that the plasma and serum levels of MMP-3 were

approximately 3- to 5-fold increased in patients with rheumatoid arthritis and systemic lupus erythematosus (SLE).

There are many other examples of elevated serum or plasma concentration levels of proteases in disease: MMP-7 in pancreatic cancer [15]; MMP-2 and -9 in breast cancer [16]; ADAM12s (A Disintegrin And Metalloprotease) and MMP-2 in preeclampsia [29-31]; and human leukocyte elastase in IBD [33]. Tables 1.1 and 1.2 provide a summary of the various enzymes that are elevated in the circulation for medical conditions that are discussed in Chapters 1.2 and 1.3.

Since proteases play a substantive role in so many major diseases and are correspondingly manifested in elevated levels in the serum or plasma, it is becoming increasingly important to be able to measure their levels and activities in clinical samples in order to further elucidate disease mechanisms. In the process of furthering the understanding of these disease mechanisms, it becomes possible to discover new and more effective diagnostic markers (e.g. a specific protease) and drugs (e.g. protease inhibitors) aimed at new targets. However, many of the studies briefly mentioned above utilized detection techniques (ELISA, immunohistochemistry, zymography, fluorogenic substrates) that require sample preparation. In the next few sections, previous detection methods will be discussed and motivation will be given on why it is best to avoid sample preparation.

1.4 Previous Detection and Concentration Strategies

The previous two sections (Chapters 1.2-1.3) establish the significance of protease detection in a wide variety of major diseases and medical conditions, both for biomedical research and for the development of novel disease diagnostics. With this motivation established, Chapters 1.4.1-1.4.2 discusses previous work in the detection of proteolytic enzymes (providing background for Chapter 2). These two sections review both techniques for the detection of concentration levels (e.g. western blot, ELISA) and for the detection of enzyme activity (e.g. zymography, chromogenic/fluorogenic substrates). While the subject of dissertation is a novel assay for *activity*, the concentration-based detection methods are also presented because they are predominantly what have been used in past studies quantifying proteolytic enzymes in disease. Since sample concentration techniques are often used in order to achieve improved detection limits, Chapter 1.4.3 reviews prior sample concentration strategies (providing background for Chapter 3).

1.4.1 Detection of Activity

In general, previous methods for measuring protease activity do not meet the performance parameters needed for sensitive and accurate detection in whole blood:

- One of the most common methods for detecting protease activity utilizes fluorogenic substrates [43-63] and chromogenic substrates [28, 45, 50, 64-67] (e.g. DABS-casein, Bodipy TR/FL casein (EnzCheck), etc.) (Figure 1.2). When proteolytically

cleaved, these protein or peptide substrates release chemical moieties that produce an increase in fluorescence or light absorption, respectively, at a particular wavelength. This change is subsequently recorded using a spectrofluorometer or spectrophometer, respectively.

- Another common method involves fluorescent resonant energy transfer (FRET)-based substrates [68-84]. In FRET, a fluorescent “donor” moiety in its excited state can transfer its energy by a non-radiative, long-range dipole-dipole coupling mechanism to an acceptor moiety in close proximity (typically several nm away), which will subsequently either fluoresce or dissipate the energy as heat (quenching). In FRET-based protease substrates, a peptide links a “donor” moiety and a quencher. Upon proteolytic cleavage of the peptide linker and separation of the “donor” and quencher, the previously quenched “donor” can emit fluorescent light.
- Fluorescent polarization assays depend on the fact that fluorescent compounds that are excited with plane-polarized light will emit polarized fluorescent light [85-88]. If the compound is large, it will remain relatively stationary and will emit polarized light in the same plane as the excitation light. In fluorescent polarization assays for protease activity, a fluorescence-labeled casein is used that, when proteolytic cleaved, produces smaller fluorescent cleavage products. These smaller cleavage products rotate faster in solution and produce a fluorescent emission that is less aligned in polarization with the original excitation. Thus, the degree of detected polarization correlates with protease activity.
- Zymography [89-99] is an electrophoretic technique for detecting protease activity that uses a substrate copolymerized with a polyacrylamide gel. Samples are

initially prepared in a standard SDS-PAGE treatment buffer, but without boiling or the use of a reducing agent. After electrophoresis, the gel is incubated in unbuffered Triton X-100 to remove the SDS from the gel. The gel is then incubated in an appropriate digestion buffer and subsequently stained with Amido Black or Coomassie Brilliant Blue, which stains peptides and proteins. Areas of the gel where protease activity cleaved the copolymerized substrate will receive less staining and will appear as clear bands against a darkly stained background.

While these methods can be rapid (sometimes real-time) and sensitive (as lows as pM), the detection is usually carried out in processed samples or in non-complex buffered solutions. When detecting protease directly in whole blood, the typical fluorescent detection methods discussed above experience dramatic reductions of their sensitivity in unprocessed blood because of autofluorescence, which raises background, and quenching, which lowers signal. In order to recover some of the lost sensitivity, these methods require significant sample preparation, which, as explained before is time consuming and can alter the sample (reducing the accuracy of these assays). For the particular case of zymography, the process is not only time consuming, but it clearly significantly alters the sample. The SDS treatment lyses cells and allows introduction of proteolytic enzymes previously internalized in cells into the plasma. Furthermore, this treatment denatures enzymes, some proportion of which will not renature after the removal of SDS with Triton X-100, resulting in a loss of some portion of the sample's activity. Finally, the final staining procedure is non-specific (stains all proteins/peptides), resulting in a high background for detection in blood as a result of the large population of proteins present in whole blood. In essence, all of the methods discussed above are not

adequate for rapid, sensitive, and accurate detection directly in whole blood. The subject of this dissertation, charge-changing substrates, overcomes this fundamental limitation.

Most related to the subject of this dissertation, charge-changing peptide substrates were first used for protein kinase A/C and proteinase K detection. A fluorescent net charge changing protein kinase A substrate, lissamine-kemptide, was developed by Miick et al. [100]. This substrate is positively charged prior to phosphorylation and becomes negatively charged afterward. Another similar method was the Promega's PepTag assay, which uses the net charge changing substrates (Dye)-LRRASLG for the detection of protein kinase A and (Dye)-PLSRTLSVAAK- for the detection of protein kinase C [101, 102]. These peptides undergo a change of charge from +1 to -1 upon phosphorylation. The peptides can also be digested by proteinase K to produce various products that are positively charged, neutral, and negatively charged; however, the positively charged fragments in this case make up only a minor component of the cleavage fragments. For all of these assays after application of an electric field (during electrophoresis), the unphosphorylated/uncleaved substrate (positively charged) and oppositely charged phosphorylated/cleaved substrate (negatively charged) migrate in opposite directions. This is due to the steady state velocity for charged particles under an electric field, as determined by the equation:

$$v_{ss} = qE/f \tag{3}$$

where q is the charge on the particle, E is the electric field strength, and f is the frictional coefficient. The electric field strength is determined by the equation:

$$E = V/L \quad (4)$$

where V is the voltage and L is the length between the electrodes. As a result, the signal for this assay is a band of fluorescence that migrates toward the anode, which is completely resolved from background fluorescence, a band that migrates toward the cathode. While the principles of these charge-changing substrate assays are similar to that developed in this dissertation (both involve a change in charge), it should be noted that the substrates mentioned above focused predominantly on kinase detection. Furthermore and most importantly, these methods were not demonstrated for direct detection in whole blood. The neutral and negatively charged peptide product fragments generated from these substrates cannot be easily resolved from blood components, which are predominantly negatively charged. As a result, these prior substrates could not be used for the detection of protease activity directly in whole blood.

1.4.2 Detection of Concentration Levels

Section 1.4.1 dealt primarily with detection of protease *activity*, which is generally performed in blood after significant sample preparation. This section presents common methods for the detection of *concentration* levels of protease in clinical samples:

- In a Western Blot (a.k.a. an Immunoblot), gel electrophoresis is used initially to separate native proteins by shape and size [103, 104]. These proteins are then transferred

out of the gel and onto a membrane (typically nitrocellulose or PVDF) using capillary action or electroblotting. The target protease is then detected using labeled antibodies through colorimetric, fluorescent, radioactive, or chemiluminescent detection. An example of an application of western blotting for the detection of degradative enzymes is the detection of serum MMP-2 [105].

- In an Enzyme-Linked ImmunoSorbent Assay (ELISA), the antigen (protease) in a sample is immobilized onto a solid support (usually polystyrene) either non-specifically though adsorption or specifically with another antibody (“capture” antibody) [106, 107]. Once the antigen is immobilized, it is then subsequently detected with a primary “detection” antibody. This antibody is either labeled to allow fluorescent, colorimetric, etc. detection or it can be reacted with a labeled secondary “reporter” antibody for subsequent detection. Several examples of the use of ELISA in the detection of proteolytic enzymes are the detection of plasma and serum MMP-1, -2, -3, and -9 [95, 108, 109] and the detection of serum elastase [110].
- In a Radioimmunoassay (RIA), known quantities of radioactive antigen (protease labeled with gamma-radioactive isotopes of iodine attached to tyrosine) are mixed with an antibody to that antigen, and this mixture is then added to blood samples containing unlabeled or "cold" antigen [111-113]. The “cold” and radioactive antigens subsequently compete for antibody binding sites, resulting in displacement of radioactive antigen from antibody. For higher concentrations of "cold" antigen, more radioactive antibody is displaced and a larger proportion of antibody is bound to “cold” antigen. Separation of bound and unbound antigen is accomplished using immunoprecipitation (with a second “anti-antibody”) followed by centrifugation. Then, the radioactivity of the free antigen

left in the supernatant is measured and compared against standards to determine the amount of “cold” antigen that was in the sample. RIA has been applied, for example, to the detection of immunoreactive trypsin in serum [114, 115] and immunoreactive cathodal trypsin-like enzyme in plasma [116].

- In Immunoprecipitation (IP), an antigen (protease) is precipitated out of solution using an antibody specific to that antigen [117-120]. Precipitation occurs because the agglutination of many molecules of antigen and antibody through multivalent binding leads to the production of large insoluble complexes. Quantification can be achieved through turbidimetric assays, which measure the change in light absorbed in the sample caused by the change in the number of light-scattering species in solution (due to agglutination). Quantification can also be achieved after IP by centrifugation, removal of the supernatant, and subsequent analysis of the antigen in the pellet using some of the methods mentioned above, such as Western blotting. IP has been used to detect serum lipase [121, 122].

All of above methods for detection of concentration levels of proteolytic enzymes require significant sample preparation, resulting in the same limitations of the activity-based detection techniques discussed in 1.4.1. Furthermore, there are two major disadvantages to using the above concentration level (antibody-based) detection schemes rather then detection of enzymatic activity. First, antibody interactions suffer from non-specific binding, which is greatly pronounced in a complex biological sample such as blood (unless a lot of sample preparation is performed initially to prevent this). Enzymatic substrates (which of course can still non-specifically bind) produce signal only upon substrate cleavage, which occurs specifically after binding to its target enzyme.

Second, amplification of signal in antibody-based methods, such as from colorimetric primary or secondary detection/reporter antibody, takes place regardless of whether the antibody is bound specifically or non-specifically (such as is the case with PCR). With enzymatic substrates, a molecule of an enzyme such as α-chymotrypsin can turn over thousands or more of substrate per minutes [65]. While non-specific cleavage reactions can occur, this can be eliminated by the development of sufficiently specific substrates. Thus, enzymatic substrates are superior for detection of proteolytic enzymes.

1.4.3 Sample Concentration Strategies

Since Chapter 3 discusses a novel technique for signal concentration for the whole blood protease assay developed in this dissertation, this section is included in order to discuss prior pre-concentration strategies that have been employed. These past methods were generally used in order to improve the sensitivity and resolution of chromatographic and electrophoretic separations. The most commonly and widely used method is "*stacking*," where a discontinuous electrophoretic gel system is used to rapidly concentrate the sample into thin bands or 'stacks,' which can then accumulate at the interface between the two gels prior to subsequent electrophoretic separation [123]. In the separation of anions (DNA, and most proteins), for example, stacking is accomplished by putting a highly porous "stacking gel" (e.g. 3-5% polyacrylamide gel), with a low ionic strength (e.g. 140 mM Tris/HCL) and a near neutral pH (e.g. pH 6.9), on top of a lower porosity "resolving gel" (e.g. 8-20% polyacrylamide gel), with a higher ionic strength

(e.g. 350 mM Tris/HCL) and a higher pH of 8-9 (Figure 1.4). The stacking then occurs by several mechanisms:

1. *Changes in Ionic Strength*: The lower ionic strength of the stacking gel results in a higher electric resistance and, as a result, a higher electrical field produced in this gel compared to the resolving gel. This causes the sample to have a higher mobility in the stacking gel, compared to the resolving gel, and allows the sample's components, at sufficiently high field strengths, to rush together (rather then diffuse and separate). When the sample reaches the lower ionic strength resolving gel, it slows down dramatically since the electric field strength is lower.

2. *Isotachophoresis:* When the sample is loaded into the stacking gel, it is loaded in an electrophoresis or running buffer consisting of Tris-glycine at pH 8-9. Glycine exists in the form of a mixture of anion and zwitterion at this pH: NH_3^+-CH_2-COO^- (Zwitterion) $\Rightarrow$ NH_2-CH_2-COO^- (Anion) + H^+. When the sample enters the stacking gel, which has a pH of 6.9, the buffer molecule's equilibrium is shifted towards the uncharged zwitterionic form. Since ions are required to carry current in electrophoretic systems and since most of the buffer molecules are uncharged, the current is predominantly carried by the anionic components in the sample. During electrophoresis through the stacking gel, the sample becomes sandwiched between higher mobility chloride ions and a small amount of lower mobility glycinate ions from the buffer in a phenomenon known as isotachophoresis. When the sample reaches the interface between the two gels, the glycinate ion concentration increases dramatically and becomes responsible for carrying the bulk of the current. As a result, the sandwiched sample correspondingly experiences a drop in mobility.

3. *Changes in Gel Density:* When the sample migrates through the stacking gel, its migration is unimpeded because of the gel's higher porosity. When the sample reaches the resolving gel, the small pores restrict the sample's migration into the gel and cause the sample's components to begin separation by charge and mass.

While all three mechanisms are typically employed in combination for stacking in SDS-PAGE, some methods of stacking in capillary electrophoresis and in microfluidic devices may employ only one or two of the above mechanisms, such as stacking by a change in ionic strength [124] or by isotachophoresis [125, 126].

In *isoelectric focusing* (IEF), or electrofocusing, amphoteric substances are separated based on their different isoelectric points (pI), where the pI is that pH in which the substance has a zero net charge [127]. At the pH equal to a substance's pI, the ampholyte is mainly present in its zwitterionic form and cannot migrate when exposed to an electric field. At a lower pH, the ampholyte becomes positively charged and migrates toward the cathode. At a higher pH, it becomes negatively charged and migrates toward the anode. Thus, in a linear pH gradient and under an electric field, amphoteric substances will migrate toward positions in the gradient where the pH is equal to their pI and will then cease to migrate further. After reaching this position, the substance then becomes focused into a sharp band.

In electrokinetic chromatography, "*sweeping*" can achieve up to a 5000-fold concentration through the use of charged micelles or microemulsions that sweep the analytes into a narrow zone prior to electrophoretic separation [128, 129]. Electrokinetic chromatography separates neutral and ionic species by differences in the analytes partitioning between a pseudo-stationary phase (micelle, emulsion, etc.) and an aqueous

mobile phase (which moves because of electroosmosis). When the pseudo-stationary phase moves from the cathodic buffer reservoir into the sample region, it picks up and accumulates the analytes and pushes them toward (hence the name “sweeping”) the boundary between the sample region and the separation region (where the analytes will subsequently be separated).

Finally, in this last example of electrophoretic concentration, Heller et al. achieved electrophoretic separation of DNA fragments and oligonucleuotides in dramatically short linear distances (millimeters), and, in the process, produced a micron-sized concentration of the sample [130]. Using high voltage gradients (24-33 V/mm) and highly concentrated and crosslinked homogenous polyacrylamide gels (12% T, 6% C to 35% T, 12% C, with T= total monomer percentage concentration (w/v) of the acrylamide plus crosslinker and C = percentage of the total monomer that is crosslinker), they achieved complete separation of 20-200 nL of sample in less then 2 minutes and in less then 2 mm linear distances of 1 mm diameter capillaries. They observed band widths of 18-25 μm for larger fragments (603-1353 bp) and 30-60 μm for intermediate and smaller fragments (72-310 bp). This work demonstrated thus that rapid, micron-scale electrophoretic focusing could be achieved through high voltage gradient electrophoresis in high-density polyacrylamide gels. For further literature on preconcentration techniques, see references [105, 131-138].

1.5 The Issue with Sample Preparation

Prior protease detection methods are rapid and sensitive, but only in buffers or after the sample have been processed into plasma or serum (Table 1.3). They are *not suitable* for the direct measurement of protease activity in whole blood because of the strong autofluorescence, background absorption, and light scattering resulting from the various components of blood [139]. The prior assay methods generally overcome this fundamental limitation by performing various sample preparation steps, which includes centrifugation, separation of blood cells, filtration, and the addition of anticoagulants. Unfortunately, these processing steps can considerably alter the concentrations and activities of proteases in the blood, reducing the overall assay accuracy.

For example, sample processing can lead to hemolysis [140], resulting in the subsequent escape of cytoplasmic proteases into the plasma. Furthermore, processing into serum leads to higher concentrations and activities of MMP-1, -3, -8, and -9, as compared to plasma, as a result of enhanced release and activation of these enzymes during coagulation and fibrinolysis [141-147]. Preparation of plasma often requires the use of anticoagulants such as EDTA and sodium citrate, which are chelators that can sequester the metal ions calcium and zinc in aqueous solution. These ions are important co-factors necessary for MMP activity [144, 145, 147]. As a result, measured MMP activity will be reduced in plasma samples treated with these two anticoagulants. Another anticoagulant commonly used in preparing plasma is heparin (e.g. Na^+-heparin, Li^+-heparin), which is a highly-sulfated glycosaminoglycan with the highest negative charge density of any known biological molecule [148]. The concentration and activity of MMP-9 is strongly

affected by heparin, in both a time- and dose-dependent manner, since it prevents coagulation and the subsequent enhanced release of these enzymes from leukocytes [149]. Heparin also allows the displacement of MMP-2 pro-domain, favoring zymogen activation. Finally, there is another consequence in preparing plasma and serum: the removal of potential disease-related protease activity. Many important proteases associated with abnormal blood and other cells (e.g. cell-membrane proteases) [150-153] are eliminated in the preparation of serum and plasma samples.

In addition to lowering the accuracy of the assay, sample preparation also increases the time, cost, labor requirements and the overall complexity of the assay. Moderately to highly complex assays must be carried out in Clinical Laboratory Improvement Amendments (CLIA) approved central laboratories, precluding them from any POC applications. POC tests are generally cheaper, faster, more efficient and provide results for routine analysis in minutes, while tests carried out in central clinical laboratories require many hours or, in some cases, days before a result is available [154, 155]. The ability to rapidly carry out assays in a POC setting is particularly important for cases in which a medical decision is needed quickly and speed can make the difference between life and death (e.g. during MOF). Thus, for many diagnostic applications and especially for POC settings, it is important to eliminate sample preparation and to measure protease activity directly in a whole blood sample.

1.6 A Whole Blood Protease Assay

Previous methods for protease detection require significant sample preparation and cannot perform detection directly in unprocessed whole blood due to quenching and autofluorescence from components within blood. The assay developed in this dissertation overcomes this fundamental limitation. The basic premise for this approach is to develop negatively charged fluorescence-tagged peptide substrates that produce positively charged fluorescent products upon cleavage by a target protease. During gel electrophoresis, the fluorescent positively charged cleavage fragment migrates from the sample well into the gel toward the cathode. The negatively charged (uncleaved) substrate migrates into the gel in the opposite direction toward the anode. The majority of the components of blood (e.g. cells, proteins), which are predominantly negatively charged or are too large to fit into the pores of the gel, migrate toward the anode or remain in the sample loading well, respectively. Thus, this method allows fluorescent signal produced from protease activity to be rapidly resolved from blood and detected.

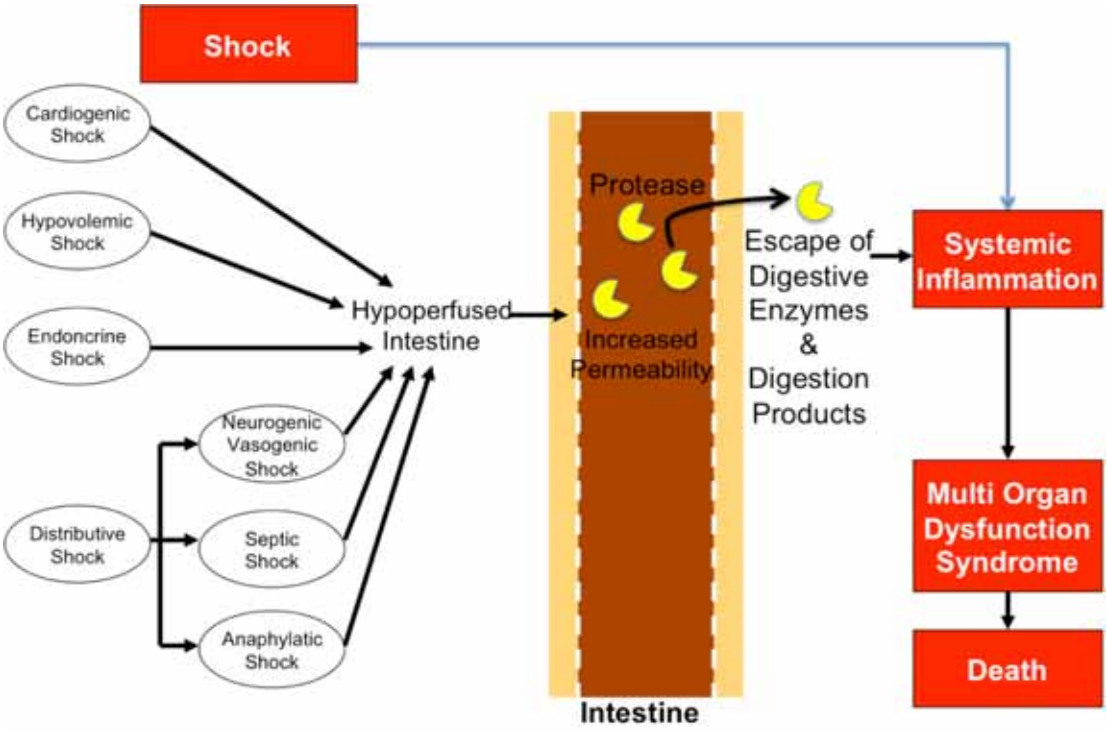

Figure 1.1: The auto-digestion hypothesis (Figure adapted with permission from Dr. Alexander Penn). During physiological shock, reduced blood flow to the intestine results in an increased endothelial and epithelial permeability of the intestinal wall. This allows the escape of pancreatic digestive enzymes from the intestinal lumen and into the circulation. The release of these enzymes, and their digestion by-products, causes systemic inflammation, which ultimately leads to highly fatal multi-organ failure.

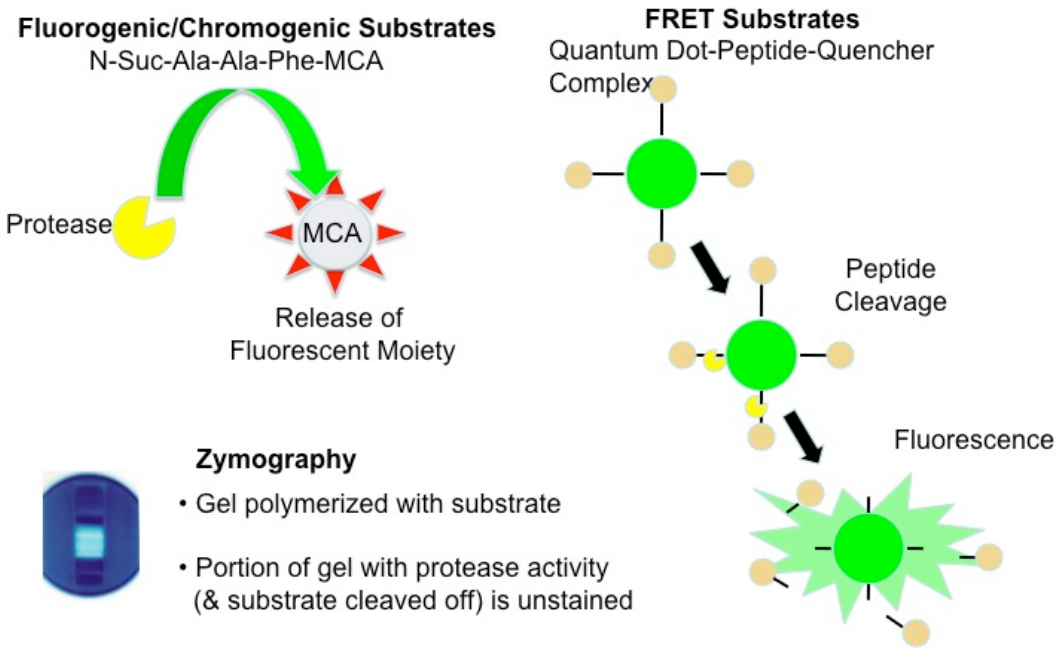

Figure 1.2: Previous methods for measuring protease activity. Upon proteolysis, fluorogenic or chromogenic substrates release a chemical moiety that results in a change in fluorescence or absorbance, respectively. For fluorescent resonant energy transfer (FRET)-based substrates, proteolytic cleavage of a linker between a fluorophore (donor) and quencher (acceptor) results in an un-quenching and subsequent restoration of the fluorescence of the donor moiety. In zymography, the protease substrate is co-polymerized into an electrophoretic gel. Protease activity is then detected after staining the gel to reveal where the substrate has been removed due to proteolysis. The image of the zymography gel is adapted from Ernst Hempelmann [156] under the Creative Commons Attribution 3.0 Unported: http://en.wikipedia.org/wiki/File:Plasmodium_knowlesi_hemoglobinase_imprint.jpg.

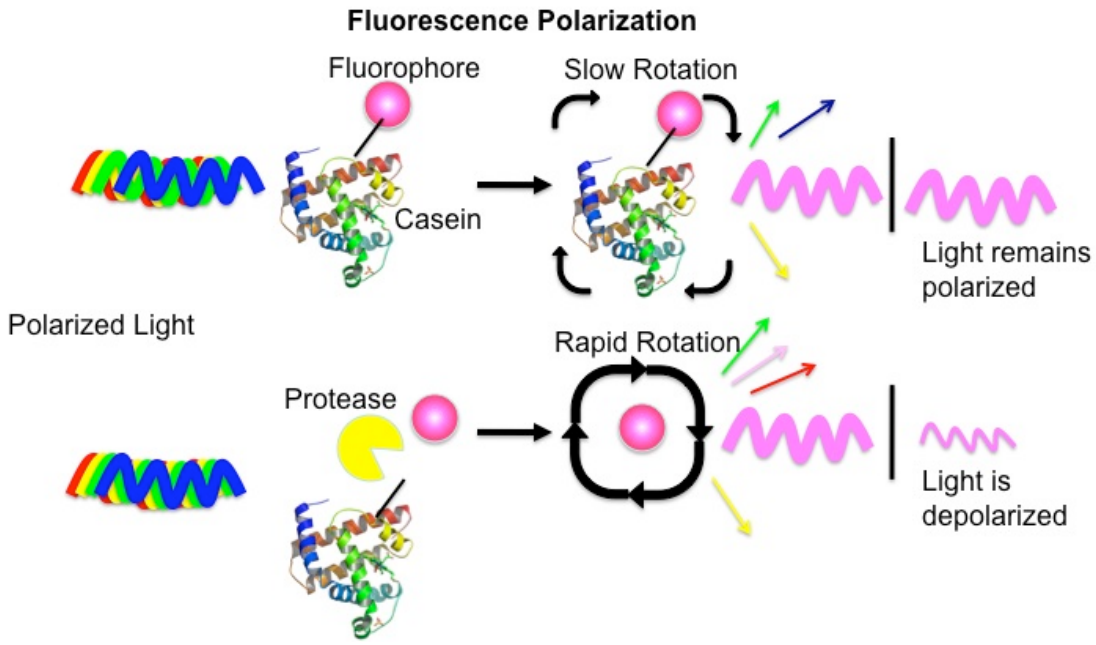

Figure 1.3: Fluorescence polarization. When a fluorescence-conjugated casein is excited by plane-polarized light, the fluorescent emission remains in the same plane because of the slow speed of rotation of the conjugate in solution. Upon cleavage of the peptide linker by an active protease, the freed fluorophore now rotates faster in solution. As a consequence, some of the fluorescence is emitted in a different plane than that of the excitation light (it is now depolarized).

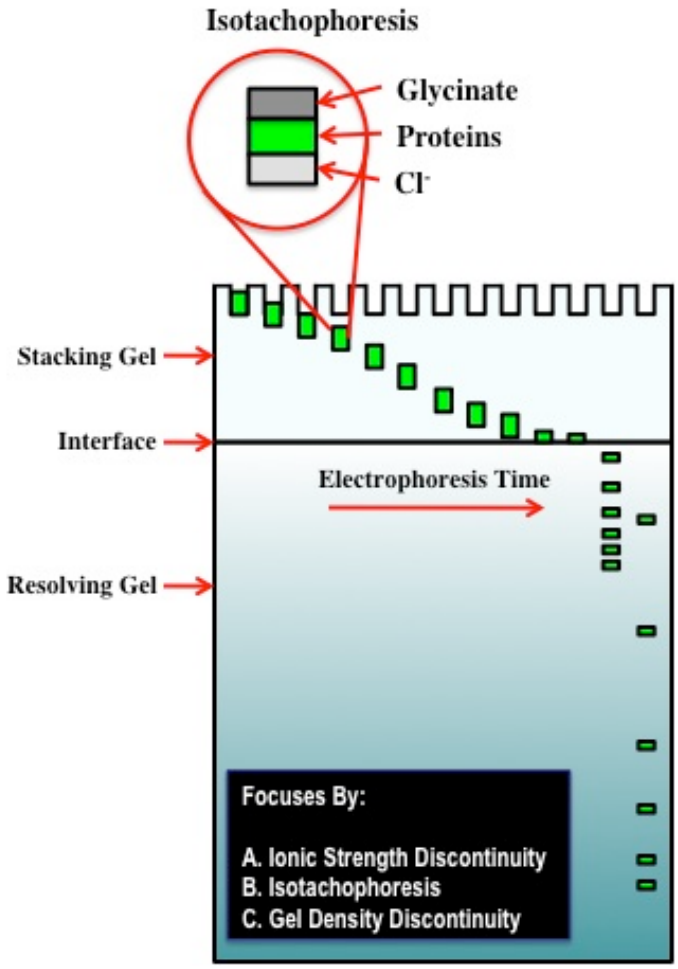

Figure 1.4: Stacking during discontinuous polyacrylamide gel electrophoresis. The sample is introduced into small wells in the stacking gel, which has a different pH, ionic strength, and polyacrylamide concentration to the resolving gel. Upon electrophoresis through the stacking gel and toward its interface with the resolving gel, proteins stack because of the discontinuities in ionic strength and gel density and by the process known as isotachophoresis.

Table 1.1: Elevated plasma and serum proteases in disease (part 1)

Medical Condition	Elevated Enzyme	Reference
Acute Coronary Syndrome	MMP-2, MMP-9	[26]
Acute/Chronic Pancreatitis	P-isoamylase, amylase, chymotrypsin, trypsin, elastase 1	[8,27,28]
Hypertension	MMP-1/9	[24]
Inflammatory Bowel Disease	Human leukocyte elastase, p-isoamylase, amylase, lipase	[32-33]
Physiological Shock	Trypsin, chymotrypsin, elastase, amylase, lipase	[172-173], Chapter 5.3.4
Preeclampsia	ADAM12s (A Disintegrin And Metalloprotease), MMP-2	[29-31]
Type 1 Diabetes	MMP-9	[20]
Type 2 Diabetes	MMP-2	[19,23]

Table 1.2: Elevated plasma and serum proteases in disease (part 2)

Medical Condition	Elevated Enzyme	Reference
Bladder Cancer	MMP-9	[10]
Breast Cancer	MMP-2, MMP-9, KLK3 (kallikrein)/PSA (prostate specific antigen), KLK5, KLK14	[6,9,10,16]
Colorectal Cancer	MMP-9	[10]
Ovarian Cancer	KLK5, KLK6, KLK8, KLK10, KLK11, KLK14	[6,9]
Pancreatic Cancer	Elastase (*possible early-stage marker)	[4,7*,8,12*, 14]
	Lipase, amylase, trypsin, elastase, tissue plasminogen activator	[13]
	MMP-7	[15]
Prostate Cancer	MMP-9, KLK2, KLK3/PSA, KLK11	[6,9,10]
Uterine Cancer (Serious Papillary)	KLK6	[6]

Table 1.3: Comparison of different protease detection methods

Method	Assay Time (Not Including Sample Prep)	Sensitivity	Sample Prep Needed?
Fluoro/Chromo-genic Substrates	seconds – 1 hr	pg-ng/ml	*YES*
FRET Substrates	seconds – hrs	pg-ng/ml	*YES*
Fluorescent Polarization	minutes	ng/ml	*YES*
Zymography	hours – 1 day	ng/ml	*YES*
Our Approach	minutes – 1 hr	pg-ng/ml	*NO*

Chapter 2:

Charge-Changing Substrates for Whole Blood Protease Detection

This is the pre-peer reviewed version of the following article: Lefkowitz RB, Marciniak JY, Hu C-M, Schmid-Schönbein GW, and Heller MJ, "An Electrophoretic Method for the Detection of Chymotrypsin and Trypsin Activity Directly in Whole Blood," *Electrophoresis* 2010, *31(2)*, 403-410 (Copyright 2009 Wiley-Blackwell), which has been published in final form at: http://www3.interscience.wiley.com/cgi-bin/fulltext/123217337/PDFSTART

2.1 Introduction

In biomedical research and clinical diagnostics, it is a major challenge to measure disease-related protease activity directly in whole blood. Present techniques for assaying protease activity require sample preparation, which makes the assays time-consuming, costly, and less accurate. Furthermore, sample preparation precludes these assays from POC applications. Chapter 2.1 introduces the use of charge-changing substrates for measuring protease activity directly in whole blood. In this simple and rapid electrophoretic method, charge-changing substrates eliminate the need for sample preparation by producing positively charged cleavage fragments that can be readily separated from the oppositely charged fluorescent substrate and blood components by electrophoresis. In this initial proof of concept, a common substrate was developed for both pancreatic α-chymotrypsin and trypsin. In whole rat blood, this substrate achieved a

limit-of-detection (LOD) of 3 ng for both α-chymotrypsin and trypsin using a 4% agarose gel. This substrate had minimal cross-reactivity with the trypsin-like proteases thrombin, plasmin, and kallikrein. Using a second trypsin-specific charge-changing substrate, it was shown that the LOD depends on the electrophoretic gel thickness and density. A detection limit of about 10-20 pg was achieved in 1X PBS using thinner higher resolution 20% and 25% polyacrylamide gels. This provided a 10- to 20-fold improvement of the LOD versus the 4% agarose gel format. Summarizing, Chapter 2 demonstrates, for the first time, that charge-changing peptide substrates enable a simple electrophoretic assay format for the measurement of protease activity in whole blood. This is an important step toward the development of novel point-of-care diagnostics.

2.2 Materials and Methods

2.2.1 Materials

Substrate 1, Ac-N-DGDAGYAGLRGAG-diamino ethyl-Bodipy FL is a fluorescence-conjugated peptide substrate for both α-chymotrypsin and trypsin that was synthesized by Bachem (King of Prussia, PA, USA). Substrate 2 is a trypsin-specific substrate Ac-N-DGDAGRAGAGK-NH_2 that was synthesized by Aapptec (Louisville, KY, USA) and subsequently labeled on the lysine residue's epsilon amine group with Bodipy FL-SE (Invitrogen, Carlsbad, CA, USA). The labeling was carried out by reacting equal volumes of 10 mg/mL of the peptide in 100 mM $NaHCO_3$ (pH 8.2) with 10 mg/mL of the fluorophore in DMSO for 1 hour (creating Substrate 2, Ac-N-

DGDAGRAGAGK(ε-Bodipy FL)-NH_2). Fluorescent absorption and emission maximum for Bodipy FL is 505 nm and 513 nm, respectively. Type IV-S bovine pancreatic α-chymotrypsin (60 units/mg); TPCK (N-tosyl-L-phenylalanyl chloromethyl ketone)--treated bovine pancreatic trypsin (13,000 units/mg); human plasma thrombin (2,955 units/mg); human plasma kallikrein (14 units/mg); human plasma plasmin (5.3 units/mg); protease inhibitor cocktail (P2714); 0.45 μm filtered human plasma treated with 3.8% trisodium citrate (P9523); high-resolution agarose (A4718); tris-borate EDTA buffer (TBE, T3913); 40% acrylamide (A4058); and calcium chloride dihydrate (C3881) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Phosphate buffered saline (PBS, 2810305) was obtained from MP Biomedicals (Solon, OH, USA). Hydrogen chloride (A-144S), ammonium persulfate (APS, BP179), and N,N,N',N'-tetramethylethylenediamine (TEMED, BP150) was obtained from Fisher Scientific (Fair Lawn, NJ, USA). N,N'-methylenebisacrylamide (Bis, 2610) was obtained from EMD Chemicals (Gibbstown, NJ). Novex pre-cast 1-mm thick 20%T 2.6%C Polyacrylamide TBE gels & Novex gel cassettes (for casting custom 1-mm thick gels) were both obtained from Invitrogen. Whole rat blood, treated with 100 USP units/ml heparin, was provided by Adam Wright/Andrew McCulloch (University of California San Diego, La Jolla, CA, USA).

2.2.2 Detection Limit in 1X PBS and Plasma

A 1 mg/mL stock solution of Substrate 1 (MW 1537.4) was prepared in 1X PBS (pH 7.8). Stock solutions of 2 mg/mL and various dilutions of α-chymotrypsin (MW 25 kDa) and trypsin (MW 23.8 kDa) were prepared in 1 mM HCl. A solution of 0.5 mg/mL

(14.4 μL) Substrate 1 was prepared in either 1X PBS (pH 7.8) or in human plasma, with 56 mM $CaCl_2$ present as a co-factor. Various concentrations (1 μL volume) of either one of the proteases, or 1 mM HCl for the negative control, were then spiked into the substrate solution in order to achieve the desired final enzyme concentrations. In this reaction mixture, the final concentration of substrate was 0.5 mg/mL and the different final concentrations of enzyme were 500, 100, 50, 30, 20, and 0 nM. The reactions were then allowed to proceed for one hour. Aliquots of 6 μL of the reaction mixtures were loaded directly into the center of 7.5x10x1 cm 4% high-resolution agarose gels in 0.5X TBE (44.5 mM Tris-Borate, 1 mM EDTA, pH 8.0), electrophoresed at 80 V for 30 min, and then imaged directly by a BioDoc-It System with a Model M-26 transilluminator (UVP, Upland, CA, USA) at an excitation of 302 nm and emission of 500-580 nm. Gels were quantified with a Storm 840 gel scanner using ImageQuant v5.2 (Molecular Dynamics, Sunnyvale, CA, USA) (fluorescence mode, high sensitivity, 100 μm pixel size, 1000V photomultiplier tube) with a 450 nm excitation filter and a 520 nm long pass emission filter. The fluorescent signal was obtained by integrating the fluorescence over the entire cleavage product band and then subtracting the background fluorescence from the negative control.

2.2.3 Detection Limit in Whole Blood

Detection limits in whole rat blood were estimated by measuring activity only from known levels of spiked α-chymotrypsin or trypsin. This requires eliminating activity caused by endogenous enzyme, whose levels are unknown. This was

accomplished by performing the cleavage reaction in buffer and then mixing with protease inhibitor-treated blood immediately prior to electrophoresis. Briefly, a solution of 0.5 mg/mL (14.4 μL) Substrate 1, containing 56 mM $CaCl_2$, was reacted with various concentrations (1 μL) of α-chymotrypsin or trypsin (or 1 mM HCl for the negative control) in 1X PBS (pH 7.8) for 1 hour. The final substrate concentration was 0.5 mg/mL and the final enzyme concentrations were: 500, 100, 50, 30, 20, and 0 nM. Heparinized whole rat blood was treated with an equal volume of 10X protease inhibitor cocktail and allowed to incubate during that same hour. The buffered enzyme reaction mixture was then mixed with protease inhibitor-treated blood in either a 2:1 (vol/vol) ratio for α-chymotrypsin detection or in a 1:1 (vol/vol) ratio for trypsin detection. Immediately after mixing the treated blood with the reaction mixture, 6 μL aliquots were electrophoresed, visualized, and quantified as described in Section 2.2.2.

2.2.4 Specificity

Enzyme stock solutions were prepared at 0.5 mg/mL concentrations: bovine pancreatic trypsin and α-chymotrypsin in 1 mM HCl; human plasma kallikrein in 20 mM Tris-HCl (pH 7.8), with 100 mM NaCl; and human plasma thrombin and plasmin in 1X PBS (pH 7.8). Ten-fold dilutions of these enzyme solutions were then obtained after dilution in 1 mM HCl. A solution of 0.5 mg/mL (14.4 μL) Substrate 1 was prepared in 1X PBS (pH 7.8), with 56 mM $CaCl_2$. In individual reaction tubes, enzyme or 1 mM HCl (negative control) was added (in a volume of 1 μL) to bring the final substrate and enzyme concentrations to approximately 0.5 mg/mL and 3μg/mL, respectively. After the

reaction proceeded for 1 hour, 6 μL aliquots were electrophoresed, visualized, and quantified as described in Section 2.2.2.

2.2.5 Kinetics

Final concentrations of 50 μg/mL of Substrate 1 and 70 μg/mL of bovine pancreatic α-chymotrypsin were reacted in 1X PBS (pH 7.8), with 56 mM $CaCl_2$ present as a co-factor. After quickly preparing this reaction mixture, the fluorescent emission was immediately recorded at 512 nm for an excitation of 450 nm for 30 minutes with a LS 55 spectrofluorometer (Perkin Elmer, Waltham, MA) (21ºC, 100 nm/min scan rate, 5 nm excitation and emission slit widths). This entire procedure was repeated for bovine pancreatic trypsin. Following the methods of Felber [71], the Michaelis-Menten kinetics ratio K_{cat}/K_m was directly estimated by using a first-order analysis that assumes that the concentration of the substrate is sufficiently smaller than K_m ($[S] << K_m$). A derivation of the formula for estimating K_{cat}/K_m is included in Appendix 1.

2.2.6. Trypsin Detection in Multiple Gel Formats

Various concentrations (1 μL volume) of trypsin in 1 mM HCl (or 1 mM HCl for the negative control) were spiked into a solution of 0.5 mg/mL (14.4 μL volume) Substrate 2 (MW 1293.1) in 1X PBS (pH 7.8) and allowed to react for 1 hour. The final substrate concentration was 0.5 mg/mL. Then, aliquots of 6 μL of the reaction mixtures were loaded directly into one of three gel formats: (Format 1) a 7.5x10x1 cm 4% high-

resolution horizontal agarose gel, (Format 2) a 8x8x0.1 cm pre-cast Novex 20%T 2.6%C vertical polyacrylamide TBE gel, or (Format 3) a 8x8x0.1 cm 25%T 6%C vertical polyacrylamide gel cast into a Novex gel cassette. The final enzyme concentrations tested were: 500, 100, 50, 30, 20, and 0 nM in Format 1; 50, 30, 10, 7, 3, and 0 nM in Format 2; and 50, 30, 10, 7, 3, 2, 0.9, 0.4, and 0 nM in Format 3. The horizontal agarose gel was loaded in the center of the gel, while the vertical polyacrylamide gels were loaded at the top. The corresponding electrophoresis conditions, voltage and run time, for those three formats were: 80V for 30 minutes for the agarose gel (Format 1) and 300V for 10 minutes for the polyacrylamide gels (Formats 2 and 3). In all cases, the running buffer was 0.5X TBE. In the final step, these gels were visualized and quantified as described in Section 2.2.2.

2.3 Results and Discussion

Charge-changing Substrate 1, Ac-N-Asp-Gly-Asp-Ala-Gly-Tyr-Ala-Gly-Leu-Arg-Gly-Ala-Gly-diamino-ethyl-Bodipy FL, was developed as an initial substrate for detecting the activity of both pancreatic serine proteases α-chymotrypsin and trypsin directly in whole blood. Substrate 1, which has a net charge of -1, is cleaved at the tyrosine-alanine bond by α-chymotrypsin and at the arginine-glycine bond by trypsin (see Table 2.1). Upon cleavage, Substrate 1 produces a fluorescent cleavage product that has a net charge of +2 for α-chymotrypsin cleavage, or +1 for trypsin cleavage. In an electrophoretic field, these positively charged fluorescent cleavage products can be easily separated from oppositely charged (uncleaved) substrate and blood background

contributors (red and white cells, heme, plasma proteins, etc.), facilitating the detection of proteases directly in whole blood without prior sample preparation. This basic sample to answer assay process is shown in Figure 2.1.

2.3.1 Detection Limit in 1X PBS

We first validated that the fluorescent peptide Substrate 1 was specific for α-chymotrypsin and trypsin by performing reactions between the substrate and the enzymes in 1X PBS. Various concentrations of the enzymes were reacted with Substrate 1 for 1 hour and then aliquots of the reactions were loaded into an agarose gel and electrophoresed. Horizontal agarose gel formats were used in these initial studies because they could be loaded with sample in the center of the gel and could show the relative separation of all components in the samples (blood components, intact substrate and its cleavage products). Gel i in Figures 2.2a and 2.3a shows the typical results produced in experiments for determining the detection limit of spiked α-chymotrypsin and trypsin in 1X PBS. Each pair of gel lanes was loaded with a reaction mixture of Substrate 1 and a different concentration of enzyme, progressing from the highest concentration on the left-hand side of the gel to the negative control on the right-hand side. For the negative control (lanes 11-12), minimal positively charged fragments were detected, and most of the fluorescence migrated toward the anode (substrate is negatively charged). As the amount of enzyme reacting with the substrate is increased (moving leftward from lanes 9-10 to lanes 1-2), more substrate is converted to product, resulting in an increasing amount of fluorescence migrating toward the cathode. The low amount of neutral to positively

charged fluorescence observed close to the wells in most lanes is most likely attributed to substrate impurity. True signal due to hydrolysis by target protease can readily be distinguished from this background impurity by sufficient electrophoresis, as is particularly evident in Gel i in Figure 2.2a. The corresponding standard curves for α-chymotrypsin and trypsin in 1X PBS are shown as Curve i in Figures 2.2b and 2.3b, respectively. Following IUPAC standards [157], the detection limit of spiked enzyme was estimated as three standard deviations from background as approximately 0.2 ng and 0.6 ng, respectively.

2.3.2 Detection Limit in Human Plasma

Before demonstrating feasibility in whole blood, we carried out the detection in a less complex biological sample, human plasma. Gel ii of Figures 2.2a and 2.3a show the typical results produced in experiments for determining the detection limit of spiked α-chymotrypsin and trypsin in human plasma. For chymotrypsin detection, the results of the human plasma experiment (Gel ii of Figure 2.2a) are quite comparable to the 1X PBS experiment (Gel i of Figure 2.2a). For trypsin detection, however, larger differences in signal were observed between human plasma (Gel ii of Figure 2.3a) and 1X PBS (Gel i of Figure 2.3a). These differences in the detection signal (the fluorescence of the cleavage product band) are most likely attributed to the presence of endogenous protease and protease inhibitors in human plasma, which can increase or decrease the amount of enzyme activity detected, respectively. The standard curves for α-chymotrypsin and trypsin in human plasma are shown as Curve ii in Figures 2.2b and 2.3b, respectively. In

both 1X PBS and plasma, the fluorescence of the cleavage product band is distinguishable from the negative control for all concentrations of enzyme tested. The detection limit of spiked enzyme was estimated to be 0.3 ng for α-chymotrypsin and 0.3 ng for trypsin, respectively. The close proximity of the detection limits in 1X PBS and in human plasma confirms that the positively charged proteolysis products are effectively separated from most potential interfering background contributions in plasma.

2.3.3 Detection Limit in Whole Blood

Our next goal was to estimate the detection limit in whole blood. Since this requires standards of known concentrations of enzyme and since we have observed endogenous trypsin-like activity in normal blood samples (un-published results) and others have observed the activity in human plasma [28], it was decided to first perform the reactions in 1X PBS and then mix the reactions products with protease inhibitor-treated whole rat blood prior to electrophoresis. By performing the protocol in this manner, the cleavage of the substrate could mostly be attributed to known levels of enzyme spiked into the reaction rather than the unknown levels of trypsin-like activity present in normal blood samples. More specifically, this experiment is key in demonstrating that the fluorescent peptide product fragments can quantitatively be recovered from the whole blood sample. Gel iii in Figures 2.2a and 2.3a show detection, directly in whole rat blood, of fluorescent cleavage products generated from reactions with various amounts of α-chymotrypsin and trypsin, respectively. The corresponding standard curves for α-chymotrypsin and trypsin in rat blood are shown as Curve iii in

Figures 2.2b and 2.3b. As was the case in 1X PBS and in plasma, the fluorescence of the cleavage product band is distinguishable from the negative control for all concentrations tested, showing that this method can retain good detection characteristics in whole blood. As before, differences in signal between 1X PBS and whole rat blood are likely due to the presence of endogenous protease and protease inhibitors present in whole rat blood. The detection limit of spiked enzyme obtained by this protocol for both enzymes was approximately 3 ng.

2.3.4 Substrate Specificity and Kinetics

To better characterize the specificity of Substrate 1, the substrate's cross-reactivity was tested with thrombin, plasmin and kallikrein, which are trypsin-like blood proteases involved in coagulation and fibrinolysis. The results in Figure 2.4a, show approximately an order of magnitude higher signal for the intended targets α-chymotrypsin (i) and trypsin (ii) than for the other three enzymes (iii-vi), confirming that this substrate is fairly specific. In another experiment of this study, the kinetics of Substrate 1 were characterized by directly estimating the ratio of the Michaelis-Menten kinetics constants k_{cat} and K_m, $k_{cat}/K_{m,}$ using a first order kinetics analysis that assumes that the concentration of the substrate is negligibly small in comparison with the value of K_m. Appendix 1 provides the derivation of the formula for estimating k_{cat}/K_m. We measured the ratio rather than the individual constants because we had a limited supply of the substrate. Without knowing K_m (and being unable to verify that [S] << Km), the measured k_{cat}/K_m should be considered a rough estimate. From the kinetics curves shown

in Figure 2.4b for α-chymotrypsin (i) and for trypsin (ii), we derived a k_{cat}/K_m ratio of 2000 s^{-1} M^{-1} and 800 s^{-1} M^{-1} for the respective enzymes.

2.3.5 Trypsin Detection in Multiple Gel Formats

The ability to detect low levels of protease activity directly in whole blood is a major advancement. The results shown in Figures 2.2 and 2.3 (Gel and Curve iii) show direct detection of signal generated from as low as 3 ng of spiked protease in whole blood using a 90-minute protocol and a 6 μL sample. This is achieved without the sample preparation generally required by current techniques. While detecting 3 ng of enzyme from 6 μL loaded into a gel is equivalent to detecting 500 ng/mL enzyme in solution, which is higher than the reference levels for trypsin of 15-60 ng/mL in human blood [28], it is important to note that this detection level was achieved using relatively thick gel formats (1-cm thick horizontal agarose gel). The final experiments using trypsin-specific Substrate 2 and thinner high-resolution vertical polyacrylamide gels demonstrates greatly improved detection limits.

Substrate 2, Ac-N-Asp-Gly-Asp-Ala-Gly-Arg-Ala-Gly-Ala-Gly-Lys(ε-Bodipy FL)-NH_2, has a net charge of -1 and is cleaved at the arginine-alanine bond by trypsin to produce a fluorescent cleavage product with a net charge of +1 (see Table 2.1). In Figure 2.5, Substrate 2 is used to detection trypsin in 1X PBS using three different gel formats. Figure 2.5a first shows the results for detection of trypsin using the same 1-cm thick 4% agarose horizontal gel format and electrophoresis conditions (30 minute run time) as in the earlier experiments. Since this is a center-loaded horizontal gel, fluorescent bands can

be observed from both the intact substrate (migrating toward the anode) and the cleavage product (migrating toward the cathode). Figure 2.5b shows results using 1-mm thick 20% polyacrylamide gel (10 minute run time) and Figures 2.5c and 2.5d show results using a 1-mm thick 25% polyacrylamide gel (10 minute run time). In these vertical gel-formats, which are loaded with samples at the top of the gel, only the fluorescent cleavage product migrates into the gel while the background contributions (intact substrate and blood components) run into the buffer. Thus, only a single fluorescent band pertaining to the cleavage product is observed in these gels. For the agarose gel, the detection limit was about 200 pg (which, for 6 μL sample loading, corresponds to detection of ~40 ng/mL). For the thinner, denser 20%T 2.6%C polyacrylamide gel, the detection limit was about 20 pg (~ 4 ng/mL). Increasing the gel density further to 25%T 6%C improved the detection limit to about 10 pg (~2 ng/mL). These results show that relatively simple improvements allow the detection limits for this assay to reach the clinically relevant baseline level for trypsin, which is 15-60 ng/mL [28]. Another simple way to improve the detection levels is to increase the sample loading. The 1-mm gels in this chapter were loaded with only 6 μL of reaction mixture. Increasing loading to this gel's capacity of 20 μL could potentially allow a further 3-fold improvement. As these detection limits continue to be improved, it should finally be noted that the overall assay time could also be reduced significantly as less reaction time is needed to reach the desired detection limits.

2.4 Concluding Remarks

In this study, specific net charge-changing fluorescent substrates were designed, synthesized and tested for the purpose of detecting α-chymotrypsin and trypsin, which are important biomarkers for a number of diseases. When used in simple electrophoretic formats, these unique substrates eliminate the need for sample preparation and allow enzyme activity to be determined directly in whole blood samples. Overall, the detection level for trypsin-specific Substrate 2, using high-resolution polyacrylamide gel formats, is well within clinically relevant levels [28]. Since the protocol presented here removes the background contributions from blood and the (uncleaved) substrate, further improvement in sensitivity using microgel formats and better epifluorescent detection systems will allow total assay time to be reduced to minutes (work in progress). These initial results also serve as a basis for the development of a number of other unique charge-changing substrates for detecting a variety of important proteases (matrix metalloproteases, lipases, amylases and nucleases). Most importantly, the ability to directly use a blood sample, with no sample preparation, is the first step in overcoming one of the major limitations in developing viable and cost-effective POC diagnostic systems.

2.5 Acknowledgements

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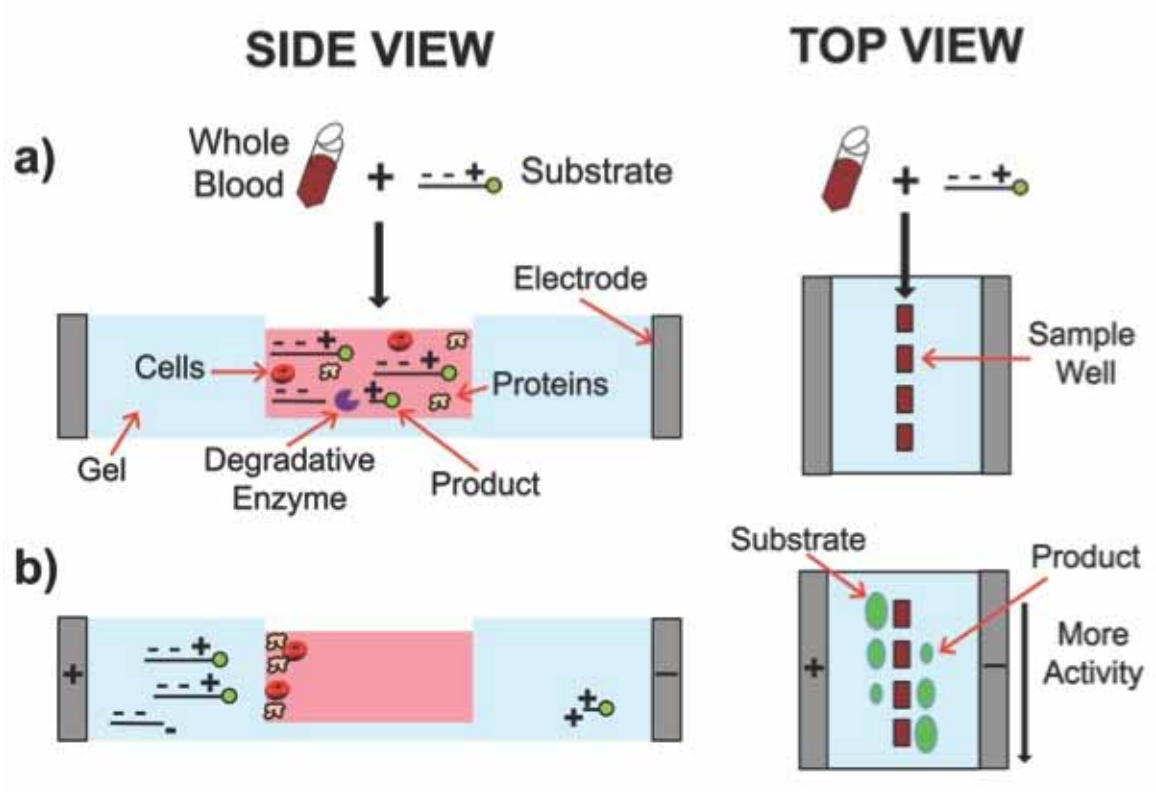

Figure 2.1: Schematic of whole blood protease assay using a charge-changing substrate. (a) The substrate is added to a whole blood sample, where it then reacts with target enzyme to produce a positively charged fluorescent signal. The mixture is then loaded into the well of an electrophoretic gel. (b) During electrophoresis, negatively charged uncleaved substrate, blood cells, and plasma proteins migrate toward the anode, or, if too large to migrate into the gel's pores, remain within the sample loading well. The fluorescent signal, which is positively charged, migrates in the opposite direction and is resolved from blood components.

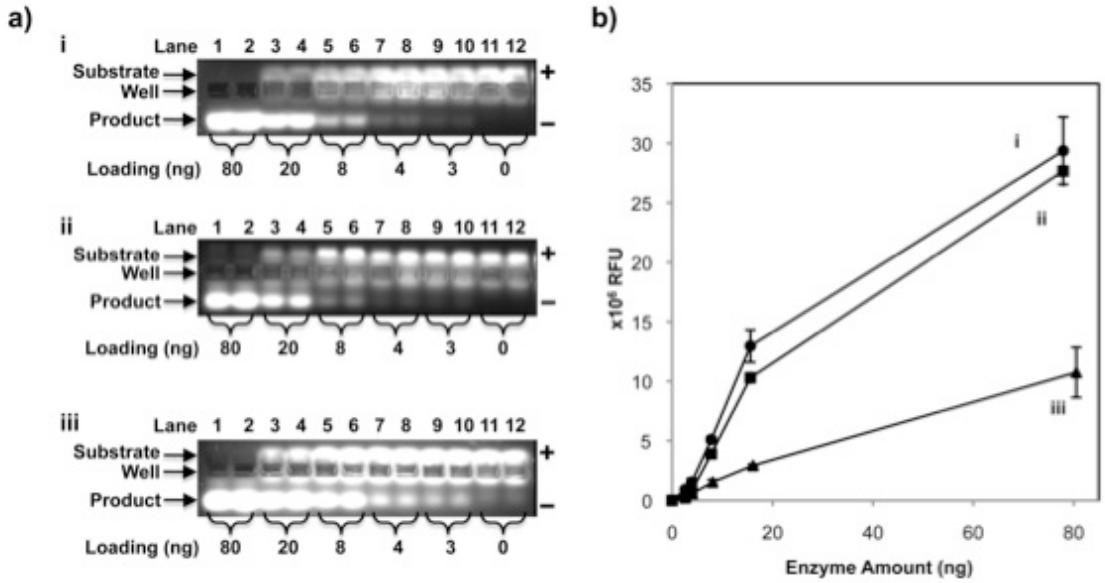

Figure 2.2: Detection of α-chymotrypsin in 1X PBS, human plasma, and whole rat blood with Substrate 1. (a) Agarose gel electrophoresis patterns generated from a 1 hour reaction of 0.5 mg/mL of Substrate 1 with several different concentrations of α-chymotrypsin in (i) 1X PBS, (ii) human plasma, and (iii) whole rat blood. Gel loading was at 6 μL/ well. The amount of enzyme loaded into the gels is designated beneath the gel. The '+' and '-' denote the respective positions of the anode and cathode and the arrows designate the positions of the sample loading well, the intact substrate band, and the cleavage product band (the signal). Negatively charged substrate migrates toward the anode, while the fluorescent cleavage product migrates toward the cathode. (b) Enzyme activity standard curves corresponding to gels in (a), with the signal reported in millions of relative fluorescence units (RFU).

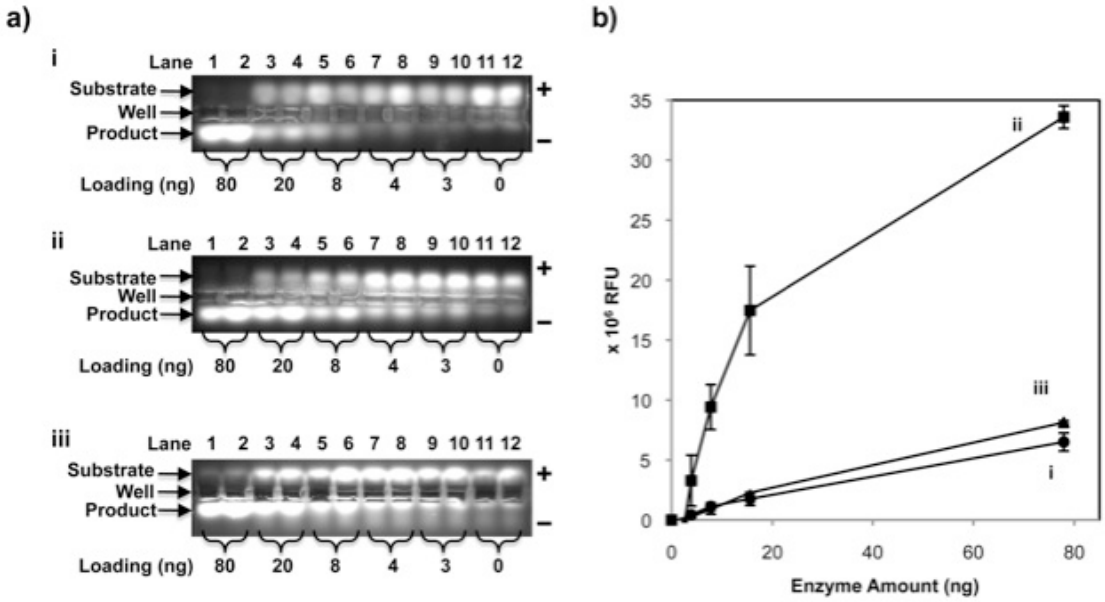

Figure 2.3: Detection of trypsin in 1X PBS, human plasma, and whole rat blood with Substrate 1. (a) Agarose gel electrophoresis patterns generated from a 1 hour reaction of 0.5 mg/mL of Substrate 1 with several different concentrations of trypsin in (i) 1X PBS, (ii) human plasma, and (iii) whole rat blood. Gel loading was at 6 μL/ well. (b) Enzyme activity standard curves corresponding to gels in (a), with the signal reported in millions of relative fluorescence units (RFU).

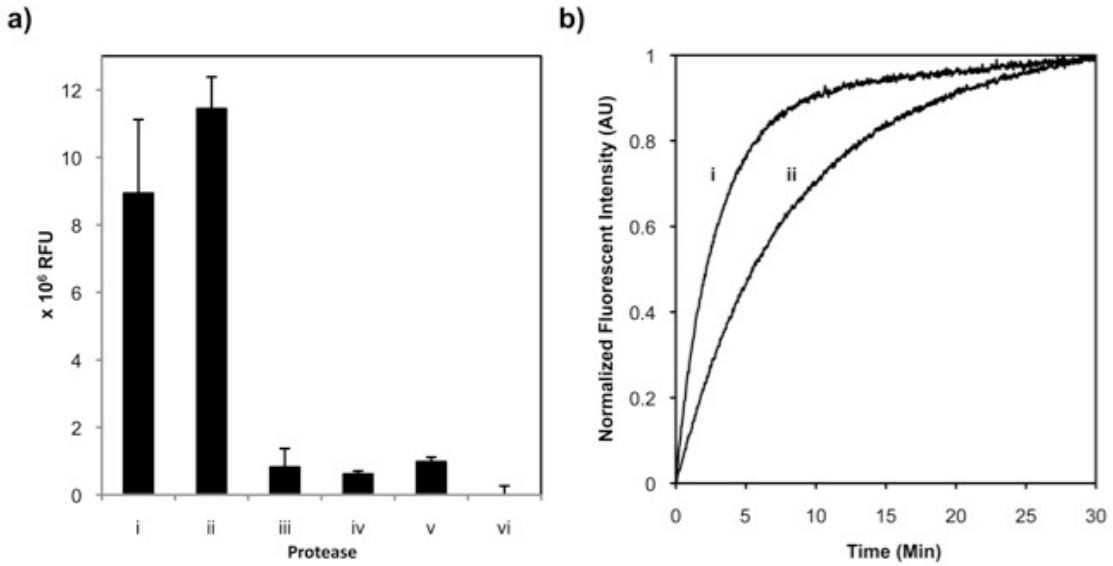

Figure 2.4: Specificity and kinetics of Substrate 1. (a) Fluorescent intensities obtained for 1 hour reactions at 21ºC between 0.5 mg/mL of Substrate 1 and 3 μg/mL of (i) α-chymotrypsin, (ii) trypsin, (iii) thrombin, (iv) plasmin, (v) kallikrein, and (vi) a negative control in 1X PBS. Gel loading was at 6 μL/ well. (b) First order kinetics for hydrolysis of Substrate 1 by (i) α-chymotrypsin and by (ii) trypsin. Reactions between 50 μg/mL of Substrate 1 and 70 μg/mL of enzyme were continuously monitored over the course of 30 minutes by recording the fluorescent emission at 512 nm for an excitation of 450 nm.

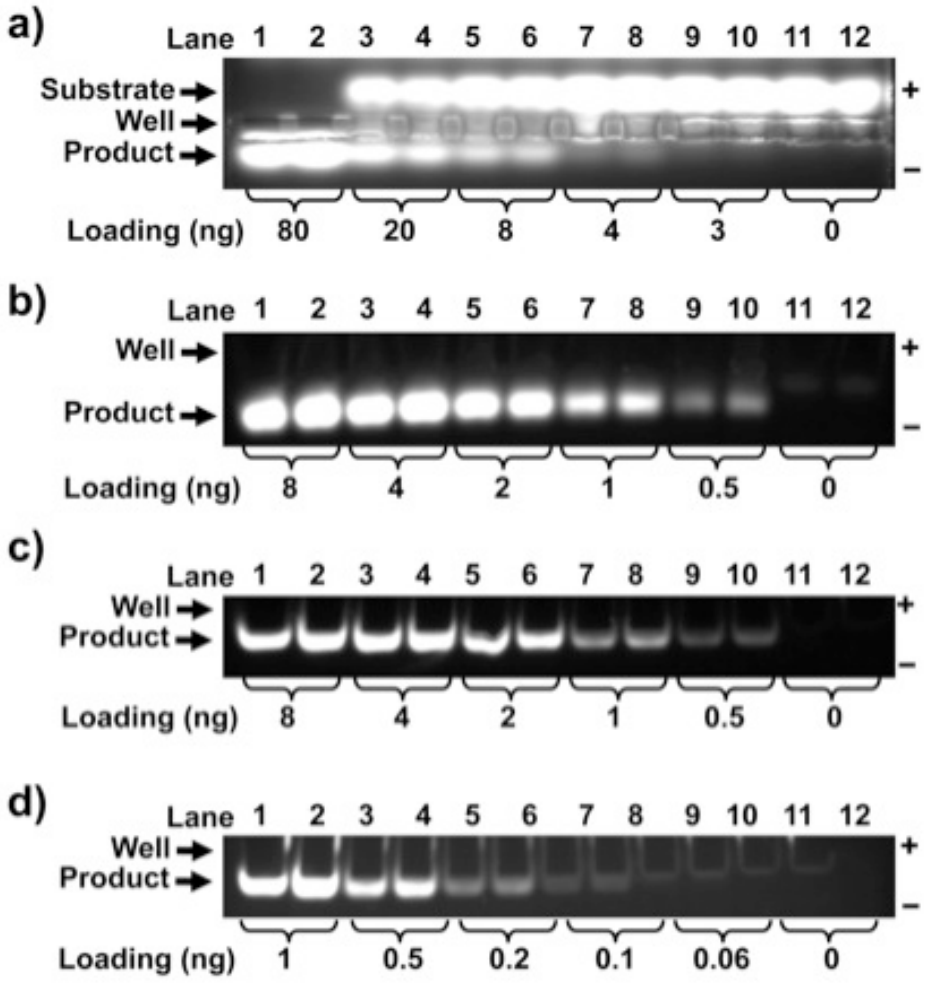

Figure 2.5: Detection of trypsin in 1X PBS with Substrate 2 in various gel formats. In each case, 0.5 mg/mL of Substrate 2 was reacted for 1 hour with several different concentrations of trypsin in 1X PBS blood and then loaded into a gel at 6 μL/well. Electrophoresis was carried out in the following gel formats: (a) 1-cm thick 4% agarose gel (b) 1-mm thick 20% polyacrylamide gel and (c)-(d) 1-mm thick 25% polyacrylamide gel (where (c) shows higher concentrations of trypsin then (d)).

Table 2.1: Amino acid sequences of Substrates 1 and 2 and their cleavage products

Peptide	Sequence (N-terminal to C-terminal)	Net charge
Substrate 1	Acetyl-N-$D^{-}GD^{-}AGYAGLR^{+}GAG$-diamino ethyl-Bodipy FL	−1
Cleavage fragments		
α-Chymotrypsin		
N-terminal	Acetyl-N-$D^{-}GD^{-}AGY$-O^{-}	−3
C-terminal	H_3N^{+}-$AGLR^{+}GAG$- diamino ethyl-Bodipy FL	+2
Trypsin		
N-terminal	Acetyl-N-$D^{-}GD^{-}AGYAGLR^{+}$-O^{-}	−2
C-terminal	H_3N^{+}-GAG- diamino ethyl-Bodipy FL	+1
Substrate 2	Acetyl-N-$D^{-}GD^{-}AGR^{+}AGAGK$(ε amino-Bodipy FL)-NH_2	−1
Cleavage fragments		
Trypsin		
N-terminal	Acetyl-N-$D^{-}GD^{-}AGR^{+}$-O^{-}	−2
C-terminal	H_3N^{+}- AGAGK(ε amino-Bodipy FL)-NH_2	+1

Chapter 2, in part, is a reprint of the material as it appears in Electrophoresis: Lefkowitz RB, Marciniak JY, Hu C-M, Schmid-Schönbein GW, and Heller MJ, “An Electrophoretic Method for the Detection of Chymotrypsin and Trypsin Activity Directly in Whole Blood,” *Electrophoresis* 2010, *31(2)*, 403-410 (publisher Wiley-Blackwell). The dissertation author was the primary investigator and author of this paper.

Chapter 3:

Polyanionic Focusing Gel Electrophoresis

This is the pre-peer reviewed version of the following article: Lefkowitz RB, Schmid-Schönbein GW, and Heller MJ, "Whole Blood Assay for Trypsin Activity Using Polyanionic Focusing Gel Electrophoresis," *Electrophoresis* 2010, *31*(*14*), 2442-2451 (Copyright 2010 Wiley-Blackwell).

3.1 Introduction

Chapter 2 demonstrates that charge-changing substrates enable a simple and rapid electrophoretic assay for the measurement of protease activity directly in unprocessed whole blood samples. In that chapter, two substrates were developed: (1) a common substrate for both α-chymotrypsin and trypsin and (2) a trypsin-specific substrate. Using the latter substrate, the goal of Chapter 3 is to improve the LOD of trypsin in order to achieve *clinically relevant* detection in whole blood. Trypsin, or its inactive precursor trypsinogen, has been shown to be abnormally elevated in the serum for both acute and chronic pancreatitis [8, 28, 158, 159] and in various forms of cancer, including pancreatic, gastric, ovarian, and biliary tract cancers [159]. Furthermore, trypsin is implicated in the progression of colorectal cancer [160] and has been used to screen for cystic fibrosis in newborns [161]. Thus, the measurement of trypsin activity in blood is clearly important for both the development of novel point of care (POC) diagnostics and for biomedical research. However, it is not sufficient to simply be able to detect any level

of protease activity in whole blood. For a protease assay to be viable for disease diagnostics and for biomedical research, it is necessary that the assay can achieve detection of clinically relevant levels (both normal and disease levels) of protease activity.

The goal of Chapter 3 is to improve the assay developed in Chapter 2 in order to achieve clinically relevant detection of trypsin. This is accomplished by using a novel and simple electrophoretic method: polyanionic focusing gel electrophoresis. In this approach, a polyacrylamide gel is doped with poly-L-glutamic acid. The resulting gel can focus the fluorescent cleavage product, markedly improving the LOD of the charge-changing substrate assay. An LOD of 2 pg in 6 μL (0.3 ng/ml) in whole human blood was achieved after a 1-hour reaction of enzyme and substrate followed by 10 minutes of electrophoresis. This is 50- to 200-fold better than the estimated reference levels for trypsin (15-60 ng/ml [28]) in blood. This straightforward technique now allows for the rapid measurement of clinically relevant levels of trypsin activity in microliter volumes of whole blood, providing a useful tool for the development of novel point-of-care diagnostics. While this chapter only demonstrates this improved detection for trypsin, the same straightforward electrophoretic technique can be applied to improve the detection of other proteases (see Chapter 4).

3.2 Materials and Methods

3.2.1 Materials

The trypsin substrate Ac-N-DGDAGRAGAGK-NH_2 was synthesized by Aapptec (Louisville, KY, USA) and subsequently labeled on the lysine residue's epsilon amine group with Bodipy FL-SE (Invitrogen, Carlsbad, CA, USA) following the manufacturer's standard labeling protocol (see [162] or Chapter 2.2.1 for details). This formed the fluorescence-tagged substrate, Ac-N-DGDAGRAGAGK(ε-Bodipy FL)-NH_2. TPCK (N-tosyl-L-phenylalanyl chloromethyl ketone)-treated 13,000 units/mg bovine pancreatic trypsin (T8802); protease inhibitor cocktail (P2714); 20,500 MW poly-L-glutamic acid (PG1, P4761); 64,000 MW poly-L-glutamic acid (PG2, P4886); type XIV deoxyribonucleic acid sodium salt from herring testes (HMW DNA, D6898); high-resolution agarose (A4718); tris-borate EDTA buffer (TBE, T3913); and acrylamide (A3553) were obtained from Sigma-Aldrich (St. Louis, MO, USA). N,N'-methylenebisacrylamide (Bis, 2610-OP) and glycerol (GX0185-6) were obtained from EMD Chemicals (Gibbstown, NJ, USA). Ammonium persulfate (APS, BP179), N,N,N',N'-tetramethylethylenediamine (TEMED, BP150), and hydrogen chloride (A-144S), were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Novex pre-cast 1-mm thick 20%T 2.6%C polyacrylamide TBE gels & Novex gel cassettes (for casting custom 1-mm thick gels) were both obtained from Invitrogen. Phosphate buffered saline (PBS, 2810305) was obtained from MP Biomedicals (Solon, OH, USA). Peripheral blood was collected from volunteers into vacutainers containing heparin anticoagulant (BD

Biosciences, San Jose, CA, USA). Plasma was obtained from these blood samples by collecting the supernatant after centrifugation at 1600g for 15 minutes.

3.2.2 Detection in 1X PBS using Various Classical Gels Formats

A 1 mg/mL stock solution of the trypsin substrate Ac-N-DGDAGRAGAGK(ε-Bodipy FL)-NH_2 (MW 1293.1) was prepared in 1X phosphate buffered saline (1X PBS, pH 7.8). Stock solutions of 2 mg/mL and various dilutions of trypsin (MW 23.8 kDa) were prepared in 1 mM HCl. A solution of 0.5 mg/mL (14.4 μL) substrate was prepared in 1X PBS (pH 7.8). Various concentrations (1 μL volume) of trypsin, or 1 mM HCl for the negative control, were then spiked into the substrate solution in order to achieve the desired final enzyme concentrations. In this reaction mixture, the final concentration of substrate was 0.5 mg/mL. After the reaction proceeded for 1 hour, aliquots of 6 μL of the reaction mixtures were mixed with 1 μL 35% glycerol (for a final concentration of 5% glycerol) and loaded directly into one of 5 different gel formats: (Format 1) a 7.5x10x1 cm 4% high-resolution horizontal agarose gel; (Format 2) a 8x8x0.1 cm pre-cast Novex 20%T 2.6%C vertical polyacrylamide TBE gel; or (Formats 3-5) 8x8x0.1 cm custom-made vertical polyacrylamide gels with respective densities of 25%T 6%C, 35%T 12%C, and 50%T 12%C that were cast into Novex gel cassettes. The final enzyme concentrations tested were: 500, 100, 50, 30, 20, and 0 nM in Format 1; 50, 30, 10, 7, 3, and 0 nM in Format 2; and 7, 3, 2, 0.9, 0.4, and 0 nM in Formats 3-5. The corresponding electrophoresis conditions, voltage and run time, for the five formats were: 80V for 30 minutes for Format 1 and 500V for 10 minutes for Formats 2-5. In all cases, the running

buffer was 0.5X TBE (44.5 mM Tris-Borate, 1 mM EDTA, pH 8.0). After electrophoresis, the gels were imaged directly by a BioDoc-It System with a Model M-26 transilluminator (UVP, Upland, CA, USA) at an excitation of 302 nm and emission of 500-580 nm. Gels were then quantified with a Storm 840 gel scanner (Molecular Dynamics, Sunnyvale, CA, USA) (fluorescence mode, high sensitivity, 100 μm pixel size, 1000V photomultiplier tube) with a 450 nm excitation filter and a 520 nm long pass emission filter. The fluorescent signal was obtained using ImageQuant v5.2 by integrating the fluorescence over the entire cleavage product band and then subtracting the background fluorescence from the negative control.

3.2.3 Detection in 1X PBS using Focusing Gels

All focusing gels were cast in Novex gel cassettes in 0.5X TBE. Nonionic focusing gels consisted of two regions, a lower "focusing" gel and an upper "extraction gel." The lower gel was an 8x6.6x0.1 cm 25%T 8%C polyacrylamide gel that was allowed to polymerize for 1 hour at room temperature. On top of this gel, an 8x0.3x0.1 cm 12%T 6%C polyacrylamide gel was cast and allowed to polymerize for 0.5 hours. Polyanionic focusing gels consisted of three regions, a lower "filler" gel, an intermediate "focusing" gel doped with poly-L-glutamic acid, and an upper "extraction" gel. The lower gel was an 8x6.1x0.1 cm 12%T 6%C polyacrylamide gel allowed to polymerize for 1 hour. The intermediate gel was an 8x0.5x0.1 cm 25%T 8%C polyacrylamide gel doped with 0.5% (w/v) of either PG1 (lower MW poly-L-glutamic acid) or PG2 (higher MW poly-L-glutamic acid). Polymerization of this gel proceeded for 0.5 hours. The uppermost

gel was an 8x0.3x0.1 cm 12%T 6%C polyacrylamide gel that was cast and allowed to polymerize for 0.5 hours. Following polymerization, a solution of 0.5 mg/mL (14.4 μL) substrate was reacted with various concentrations (1 μL) of trypsin (or 1 mM HCl for the negative control) in 1X PBS (pH 7.8) for 1 hour (as described before in Section 3.2.2). The final substrate concentration was 0.5 mg/mL and the final enzyme concentrations were: 5000, 7, 3, 2, 0.9, 0.4, and 0 nM. The highest concentration (5000 nM) was tested simply to produce enough fluorescent signal such that the progress of the electrophoresis could be tracked by eye (either with excitation from ambient light or a handheld UV lamp). After completion of the reactions, aliquots of 6 μL of these mixtures were mixed with 1 μL 35% glycerol (for a final concentration of 5% glycerol) and loaded into these gels. Electrophoresis voltages and run times were 500V and 15 minutes for nonionic focusing gels, 500V and 8 minutes for PG1 polyanionic focusing gels, and 500V and 6.5 minutes for PG2 polyanionic focusing gels. After electrophoresis, imaging and quantification were performed as described in Section 3.2.2, except that the fluorescence was integrated only for the portion of the cleavage product band that was within the focusing gel.

3.2.4 Time Course of Signal Enhancement in Focusing Gel

HMW DNA-doped and PG1-doped focusing gels were prepared in Novex gel cassettes in the same way (same polyacrylamide gel monomer and cross-linker concentrations, gel dimensions, and polymerization time) as described in Section 3.2.3 for PG1 and PG2 focusing gels, except doping was with 0.5% (w/v) HMW DNA or 1%

(w/v) PG1 (rather than with 0.5% (w/v) PG1 or 0.5% (w/v) PG2 as before). The HMW DNA had been sheared in advance of doping by vortexing for 3 minutes. Trypsin reactions were then prepared and loaded into the gel as described in Section 3.2.2. During the 10 minutes of electrophoresis at 500V, photographs of the gel, still in the cassette, were imaged by the UVP BioDoc-It system (same system described in Section 3.2.2) at 5, 6, 8, and 10 minutes. The fluorescent signal was quantified using ImageJ v1.40g by integrating the fluorescence over the portion of the cleavage product band within the focusing gel and then subtracting the background fluorescence from the negative control.

3.2.5 Detection in Whole Blood and Plasma using Focusing Gels

Detection limits in heparinized human whole blood and in plasma extracted from those same blood samples were estimated by measuring activity only from known levels of spiked trypsin. As explained before in [162] (Chapter 2), this requires eliminating activity caused by endogenous enzyme, whose levels are unknown. This is accomplished by performing the cleavage reaction in buffer and then mixing with protease inhibitor-treated blood or plasma immediately prior to electrophoresis. Briefly, a solution of 0.5 mg/mL (14.4 μL) substrate was reacted with various concentrations (1 μL) of trypsin (or 1 mM HCl for the negative control) in 1X PBS (pH 7.8) for 1 hour. The final substrate concentration was 0.5 mg/mL and the final enzyme concentrations were: 5000, 7, 3, 2, 0.9, 0.4, and 0 nM. Heparinized whole blood and plasma extracted from those same blood samples were treated with an equal volume of 10X protease inhibitor cocktail and allowed to incubate during that same hour. The buffered enzyme reaction mixture was

then mixed with protease inhibitor-treated blood in a 1:1 (vol/vol) ratio. Immediately after mixing the treated blood with the reaction mixture, 6 μL aliquots of the resulting mixture were loaded in PG1 focusing gels that were prepared in advance as described in Section 3.2.3. After electrophoresis at 500V for 10 minutes, imaging, and quantification were then performed as described in Sections 3.2.2 and 3.2.3 (integrating fluorescence from the portion of the cleavage product band that is within the focusing gel).

3.2.6 Detection of Spiked Trypsin in Whole Blood

A 1 mg/mL solution of the trypsin substrate, in 1X PBS (pH 7.8), and selected dilutions of trypsin, in 1 mM HCL, were prepared as described in Section 3.2.2. Samples (7 μL) of human heparinized whole blood, obtained from three healthy individuals, were mixed 1:1 with 1 mg/mL (7 μL) substrate (or with 1X PBS (pH 7.8) as a “no substrate” control). Various concentrations (1 μL) of trypsin (or 1 mM HCl for the negative control) were spiked into the resulting mixture (14 μL) and allowed to react for 30 minutes. The final substrate concentration was 0.5 mg/mL. For reactions with substrate present, the final enzyme concentrations tested were: 50, 30, 10, and 0 nM. For the “no substrate” control, no trypsin was added. For comparison with spiking in 1X PBS (pH 7.8), a fourth set of reactions was performed where 1 mg/mL (7 μL) of the substrate solution was mixed 1:1 with 1X PBS (pH 7.8) (7 μL). Trypsin (1 μL) was then spiked into these samples and allowed to react for 30 minutes. In these reactions, the final substrate concentration was 0.5 mg/mL and the final enzyme concentrations tested were: 50, 30, 10, 7, 3, and 0 nM. After completion of these 4 sets of reactions, aliquots of 6 μL of these

mixtures were loaded into 4 separate 8x8x0.1 cm pre-cast Novex 20%T 2.6%C vertical polyacrylamide TBE gels. Following electrophoresis at 500V for 10 minutes, imaging, and quantification were then performed as described in Sections 3.2.2.

3.3 Results and Discussion

The sequence Ac-N-Asp-Gly-Asp-Ala-Gly-Arg-Ala-Gly-Ala-Gly-Lys(ε-Bodipy FL)-NH_2 was developed as a substrate for the detection of the pancreatic serine protease trypsin in whole blood. This charge-changing substrate (henceforth referred to simply as the "substrate") has a net charge of -1 prior to cleavage. Cleavage of the substrate by trypsin at the arginine-alanine bond produces a fluorescent cleavage product (the signal) with a net charge of +1 (Figure 3.1A). Figures 3.1B and 3.1C show the basic steps of the trypsin assay that we previously developed in our first report on this technique (see Chapter 2) [162]. The substrate is first mixed with a blood sample and allowed time to react with target enzyme in the blood and produce the positively charged cleavage fragment (Figure 3.1B). Upon electrophoresis, the fluorescent positively charged cleavage migrates into the gel, where it is readily separated from oppositely charged (uncleaved) substrate and from the predominantly negatively charged components of blood (cells, heme, plasma proteins, etc.) (Figure 3.1C). This initial gel is referred to as the "extraction gel" since it is in this gel that the signal is first removed from blood. Electrophoresis into the extraction gel facilitates detection of protease activity directly in whole blood since the components that would otherwise quench fluorescence or introduce autofluorescent background are mostly removed by electrophoresis. In this

chapter, we now introduce a straightforward improvement where we use a secondary gel, a polyanion-doped polyacrylamide “focusing gel,” to concentrate the fluorescent signal and lower the trypsin detection limit to clinically relevant levels (Figure 3.1D). Briefly, one of the main reasons for this focusing is the electrostatic interaction between the polyanionic dopant (polyglutamate) and the cationic signal (fluorescent cleavage fragment) (Figure 3.2). This interaction slows the migration of cleavage fragment and allows it to be concentrated.

3.3.1 Detection in 1X PBS using Various Classical Gels Formats

Prior to studying the use of a focusing gel, we first compared five different gel formats with different gel densities and thicknesses to determine which would provide the lowest limit-of-detection (LOD) for trypsin in 1X PBS buffer. In our prior report (Chapter 2), we compared a 1-cm thick 4% horizontal agarose gel (Format 1), a 1-mm thick 20%T 2.6%C vertical polyacrylamide gel (Format 2), and a 1-mm thick 25%T 6%C vertical polyacrylamide gel (Format 3). Following IUPAC standards [157], the LOD of spiked enzyme was estimated as three standard deviations from background. We determined that the best LOD was achieved in the thinnest, highest density gel tested (Format 3), with a LOD of 10 pg (~2 ng/mL for 6 μL sample loading) [162]. While this is good enough to detect clinically relevant baseline levels of trypsin (15-60 ng/mL, [28]), this LOD was estimated from measurements in 1X PBS. Anticipating some potential increase in the LOD in whole blood due to residual background (not removed upon electrophoresis), we wanted to extend the analysis of our previous report (Chapter 2) and

determine whether a further increase in gel density to 35%T 12%C (Format 4) or 50%T 12%C (Format 5) would further improve the LOD. Various concentrations of the enzymes were reacted with the substrate for 1 hour and then aliquots of the reactions were loaded into the gels and electrophoresed as described in Section 3.2.2. Gels i-v in Figure 3.3A shows the typical results for the detection of trypsin spiked in 1X PBS using Formats 1-5, respectively. Each pair of gel lanes was loaded with a reaction mixture of substrate and a different concentration of enzyme, progressing from the highest concentration on the left-hand side of the gel to the negative control (no enzyme added) on the right-hand side. For the negative control, minimal positively charged fragments (likely substrate impurity) were detected since most of the fluorescence migrated toward the anode (substrate is negatively charged). As the amount of enzyme reacting with the substrate is increased (moving leftward), more substrate is converted to product, resulting in an increasing amount of fluorescence migrating toward the cathode. The corresponding standard curves are shown as Curves i-v, respectively, in Figure 3.3B. The LOD was estimated to be 0.2, 0.03, 0.01, 0.02, and 0.04 ng in Formats 1-5, respectively. This corresponds to concentrations of 40, 4, 2, 3, and 7 ng/mL, respectively, for a 6 μL loading of sample in the gels. The results show that further increases in the gel density from 25% to 35% and further to 50% did not improve the LOD and, on average, actually worsened it. This is initially surprising considering that the fluorescence values appear increased in the 35% polyacrylamide gel when compared to the 25% polyacrylamide gel (Gels iii-iv of Figure 3.3A). However, it should be noted that the LOD is determined by the slope of the detection curve (m) as well as the standard deviation of background (s_b) (LOD is defined as $3s_b/m$). While m in the 35% gel is increased 1.5-fold relative to m for the 25%

gel, s_b increased 2.8-fold, resulting in an overall 1.9-fold increase in the LOD. There are two potential explanations for the higher LOD in the 35% and 50% polyacrylamide gels. First, these gels were more fragile and difficult to fabricate than the lower density polyacrylamide gels and, consequently, had a higher standard deviation of background (data not shown). Second, as the gel density is increased, the pore size decreases asymptotically such that there is a decreasing amount of gain in resolving power for electrophoretic gels beyond a certain density [163, 164]. Figures 3.4 and 3.5 provide support for this explanation as they show that there is negligible improvement in decreasing the band size for the fluorescent cleavage fragment for gels denser than 25% polyacrylamide.

3.3.2 Detection in 1X PBS using Focusing Gels

After determining that the 25% polyacrylamide gel provided the lowest LOD, the next step was to determine if a polyanion-doped 25% polyacrylamide focusing gel could further concentrate the fluorescent cleavage fragment so that we could achieve an even lower LOD. The fluorescent cleavage product would first be resolved from whole blood components by electrophoretic migration into a relatively low-density 12% polyacrylamide extraction gel. Then, similar to electrophoretic stacking, the signal would rapidly migrate through the extraction gel (analogous to a stacking gel), reach the higher density 25% polyanionic focusing gel (analogous to a resolving gel), and then concentrate upon entering the focusing gel. We hypothesize that this concentration would occur for two possible reasons that slow migration of the signal fragment and hence help

it focus: 1) electrostatic attraction between the positively charged signal and the polyanion and 2) decreased effective pore size due to the polyanion occupying space in the pores of the polyacrylamide gel (See Figure 3.2). In Figure 3.6, we compare trypsin detection in a 25% polyacrylamide focusing gel that is undoped (Figure 3.6A) with those that are either doped with a lower molecular weight (20,500 g/mole) poly-L-glutamic acid (referred to as PG1, see Figure 3.6B) or with a higher molecular weight (64,000 g/mole) poly-L-glutamic acid (referred to as PG2, see Figure 3.6C). Figures 3.6A-C includes both the quantitative fluorescent gel scan (lower image) and photos obtained from an imaging system (upper image) to help clarify the boundaries between the different gel regions. The gels have two to three regions, depending on the format: the uppermost extraction gel (for separation of signal from blood), the focusing gel just below (for concentration of fluorescent signal), and the lowermost “filler gel” present only in the formats that use polyanionic focusing gels. This last gel is a lower-density, undoped gel that is designed for economical reasons to fill up unused space at the bottom part of the gel since extraction of signal and subsequent focusing occurs within the top 1 cm of the gel. In all cases, electrophoresis is terminated when most of the positively charged cleavage fragment has migrated into the focusing gel for the lower concentrations of enzyme tested. In many of higher enzyme concentrations tested, some of the cleavage product still remains in the extraction gel because of loading effects (higher loading of enzyme produces a larger band), but electrophoresis is terminated anyway to minimize electrophoresis time and band diffusion. The corresponding detection curves are shown in Figure 3.6D. The LOD of trypsin spiked into 1X PBS with an undoped focusing gel, a PG1-doped focusing gel, and a PG2-doped focusing gel was

estimated to be on average (for 2 gel repeats) 5, 1, and 4 pg (which corresponds to 0.8, 0.2. and 0.6 ng/mL for 6 μL gel loading). Doping with the lower molecular weight PG1 (vs. PG2) improved the LOD by as much as 4-fold over simply using only a gel-density discontinuity (undoped focusing gel). Doping with the higher molecular weight PG2 did not improve the LOD as much, likely because of limitations of the 25% polyacrylamide gel to accommodate a doping with too much negative charge. We speculate that this overdoping may interfere with cross-linking of the polyacrylamide gel and result in larger pore sizes then would be expected in an undoped gel. Table 3.1 summarizes the LOD for trypsin in the various gels examined thus far.

3.3.3 Time Course of Signal Enhancement in a Focusing Gel

To visualize the concentration of the fluorescent cleavage product in a polyanion-doped focusing gel, we cast two different types of 25% polyacrylamide focusing gels, one doped with 0.5% (w/v) HMW DNA (vortex sheared) and the second doped with 1% (w/v) PG1, and then took a series of photos of the fluorescent band migration over the course of 10 minutes of electrophoresis. On top of this gel was cast the usual 12% polyacrylamide extraction gel. Figure 3.7A shows the HMW DNA-doped focusing gel and Figure 3.7B shows the PG1-doped focusing gel, with corresponding fluorescent intensities for the portion of band within the focusing gel plotted in Figures 3.7C and 3.7D. As time progresses, it is clear that the slope of the detection curve increases as fluorescence accumulates and concentrates in the focusing gel. Over the course of 5 minutes (from t=5 minutes to t=10 minutes), the fluorescence increase in the HMW

DNA-doped focusing gel ranged from approximately 4.1- to 8.9-fold, with greater increases in signal, in general, for higher concentrations of enzyme tested. In the PG1-doped focusing gel, the fluorescence increase ranged from 1.1-fold to 4.1-fold. With further optimization of the molecular weight and charge of the doped polymer as well as the polyacrylamide gel density, it is likely that this concentration can be further improved.

3.3.4 Detection in Whole Blood and Plasma using Focusing Gels

In our next experiment, the goal was to estimate the trypsin LOD in whole human blood and plasma using PG1-doped polyacrylamide focusing gels. Since this requires standards of known concentrations of enzyme and since we have observed endogenous trypsin-like activity in normal blood samples (un-published results) and others have observed the activity in human plasma and serum [8, 28, 158, 159, 161], it was decided to first perform the reactions in buffer (1X PBS) and then mix the reactions products with protease inhibitor-treated whole blood or plasma immediately prior to electrophoresis. By performing the protocol in this manner, the cleavage of the substrate is mostly attributed to the known levels of exogenous enzyme spiked into the reaction rather than the unknown levels of endogenous trypsin-like activity. As in the prior chapter (Chapter 2), this experiment demonstrates that the fluorescent peptide product fragments can quantitatively be recovered from whole blood [162]. Figures 3.8A and 3.8B show a representative gel for detection of trypsin cleavage products resolved from whole human blood (Figure 3.8A) and from human plasma extracted from those same blood samples

(Figure 3.8B). The corresponding detection curves are shown in Figure 3.8C for blood (solid lines) and for plasma (dashed lines). The LOD was estimated to be 2 and 0.6 pg (0.3 and 0.09 ng/mL), respectively, which is similar to the LOD for trypsin in 1X PBS (1 pg, 0.2 ng/mL). Detection in whole blood and plasma produces similar detection curves, with differences in signal appearing more so at the higher concentrations of enzyme, where variability is higher. Greater differences were observed when comparing the detection in plasma and blood (Figures 3.8C, solid and dashed lines) with the detection in 1X PBS (Figure 3.6D, dashed line), which has higher signal. The lower signal in blood and plasma, measured in relative fluorescence units (fluorescent signal subtracting background fluorescence of negative control), may be due to higher background present that has not been completely resolved within the relatively short period (10 minutes) of electrophoresis. This residual background (e.g. from potential positively charged blood components) may lower signal due to quenching or raise background due to autofluorescence. Further optimization of electrophoresis conditions (longer run time, higher resolution gels) may better remove such background. Nevertheless, the LOD obtained, particularly in whole blood, is noteworthy because the estimated reference levels are 15-60 ng/mL [28], which is 50- to 200-fold higher. Thus, this result clearly shows the capability to detect activity at clinically relevant levels of trypsin in whole blood.

3.3.5 Detection of Spiked Trypsin in Whole Blood

The previous experiment minimizes the contribution of unknown levels of endogenous protease activity and protease inhibition in the assay so that signal can be attributed to known concentrations of trypsin. However, it is important to note that this is not exactly the same as detection of trypsin activity originating from within a whole blood sample. Rather, the prior experiment demonstrates recovery of fluorescent signal from blood for a protease reaction that originated from buffer. There are at least three major differences between the detection of trypsin spiked into a buffer versus the detection of trypsin spiked into a whole blood sample. First, in a whole blood sample, there are endogenous protease inhibitors, such as α_1-antitrypsin and α_2-macroglobulin, that may block protease activity (and lower the detection signal). Second, there are variable levels of numerous endogenous proteases that can potentially cleave the substrate (and raise the detection signal). Some of this activity may be from the intended target (endogenous trypsin) and some may be due to non-specific cleavage, such as from trypsin-like proteases. Finally, there are co-factors (such as calcium) present in the blood that may aid in the activity of trypsin, therefore making detection more sensitive in blood than in 1X PBS (for a fixed concentration level of enzyme).

Thus, the goal of our final experiment (Figure 3.9**)** was to estimate the LOD for trypsin spiked directly in whole blood, with trypsin reacting with the substrate in the presence of endogenous protease, protease inhibitors, and co-factors. With an expected baseline activity from 15-60 ng/mL of trypsin [28] (which corresponds to 90-360 pg for 6 μL of sample), it was already sufficient to estimate the LOD with pre-cast 20%

polyacrylamide gels rather than using more sensitive PG1-doped polyacrylamide focusing gels. Gel i of Figure 3.9A shows the result for a 30 minute reaction of various concentrations of trypsin with the substrate in 1X PBS (pH 7.8). The corresponding detection curve is shown in Figure 3.9B (dashed line). In this 20% polyacrylamide gel, the LOD was estimated to be 20 pg (3 ng/mL), which is similar to the LOD for trypsin in this same gel after 1 hour reactions of trypsin and substrate (see Figure 3.3B, ii). Gels ii-iv of Figure 3.9A show the results for various concentrations of trypsin spiked directly into whole blood samples obtained from 3 healthy individuals (blood samples 1-3, respectively). In addition to the negative control (blood mixed with substrate, but with no enzyme added), an additional control was included to show signal from blood when no substrate or enzyme are added to the samples. These “no substrate” controls, consisting of whole blood half-diluted with 1X PBS (pH 7.8), were loaded into the rightmost pair of lanes (to the right of the dashed line in Gels ii-iv of Figure 3.9A). For these controls, no discernable fluorescence was detected. Thus, fluorescence signal is observed only upon addition of substrate and upon cleavage by either endogenous protease or spiked protease. The corresponding detection curves are in Figure 3.9B (solid lines). The fluorescence values after background subtraction and expression in relative fluorescence units (RFU), show higher signal for spiked trypsin in blood. This is likely due to the presence of co-factors in the blood that result in the enzyme being more active. The average LOD for these three gels was 200 pg (30 ng/mL), which falls within the range of the estimated levels of trypsin. This LOD is higher during protease spiking in whole blood as compared to spiking in 1X PBS because of the higher background contributed from baseline activity due to endogenous protease. The effect is not shown in the curves

of Figure 3.9B due to the background subtraction (in RFU). While the slope (m) of the curves in blood increases approximately 4-fold, the standard deviation of background (s_b) increases approximately 40-fold, resulting in a 10-fold higher LOD in whole blood (recall, LOD = $3s_b/m$). Despite this higher detection limit for trypsin spiked into whole blood (compared with 1X PBS), it should be noted that the detection limit still falls in the range of estimated reference levels for trypsin (15-60 ng/mL [28]). Thus, these results with protease spiked directly into whole blood confirm that it is feasible to detect clinically relevant levels of trypsin spiked into unseparated whole blood samples.

3.4 Concluding Remarks

In this study, polyanion-doped polyacrylamide focusing gels were designed and tested for the purpose of improving our recently developed charge-changing substrate technique for assaying protease activity *directly* in whole blood. In this assay, charge-changing substrates are cleaved by target enzyme to produce a positively charged cleavage fragment that can be readily separated from whole blood components by electrophoresis, thus eliminating the need for sample preparation [162]. While our previous study showed the detection of clinically relevant levels of trypsin activity in 1X PBS buffer, we now show detection limits significantly lower than those levels and in whole human blood. We achieved this using high-density polyacrylamide gels doped with poly-L-glutamic acid, which can focus the fluorescent signal generated from the charge-changing substrate assay and facilitate the detection of *clinically relevant* levels in whole blood. Further improvement in the detection limit can be obtained with the

incorporation of thinner microgel formats and more sensitive fluorescent labeling (e.g. nanoparticles). The simple gel format can be broadly applied to enhance charge-changing substrate detection of a number of proteases (e.g. chymotrypsin, MMPs, etc.) and other proteases such as lipases, amylases and nucleases. A variety of further miniaturized and multiplexed lab-on-chip assay formats can also be easily envisioned. The ability to rapidly measure clinically relevant levels of protease activity directly in whole blood, without any sample preparation, overcomes one of the major limitations in developing viable and cost-effective POC tests that measure protease activity.

3.5 Acknowledgements

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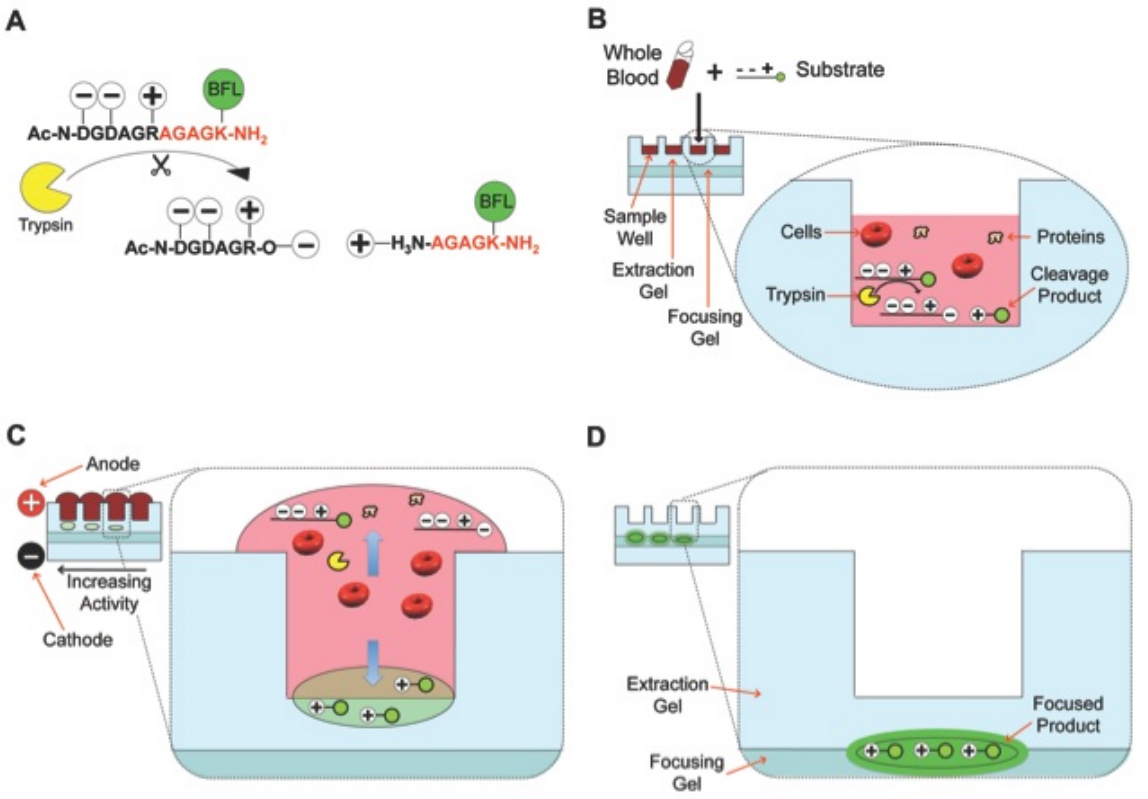

Figure 3.1: Schematic of a whole blood assay of trypsin that utilizes a charge-changing substrate. (A) Proteolysis of the negatively charged substrate by its target enzyme trypsin generates a fluorescent positively charged cleavage product. (B) The substrate is added to a whole blood sample, where it reacts with trypsin in the blood to generate the signal, the fluorescent cleavage product. This mixture is then loaded into the well of an electrophoretic gel consisting of two regions, an “extraction gel” for separation of the signal from whole blood and a “focusing gel” for subsequent concentration of the signal. (C) During electrophoresis, blood cells, plasma proteins, and negatively charged uncleaved substrate migrate toward the anode, or, if too large to migrate into the gel’s pores, remain within the sample loading well. The positively charged fluorescent signal migrates in the opposite direction into the extraction gel and is resolved from blood components. (D) Subsequent electrophoresis into the denser, polyanion-doped focusing gel concentrates the fluorescent signal and facilitates lower detection limits.

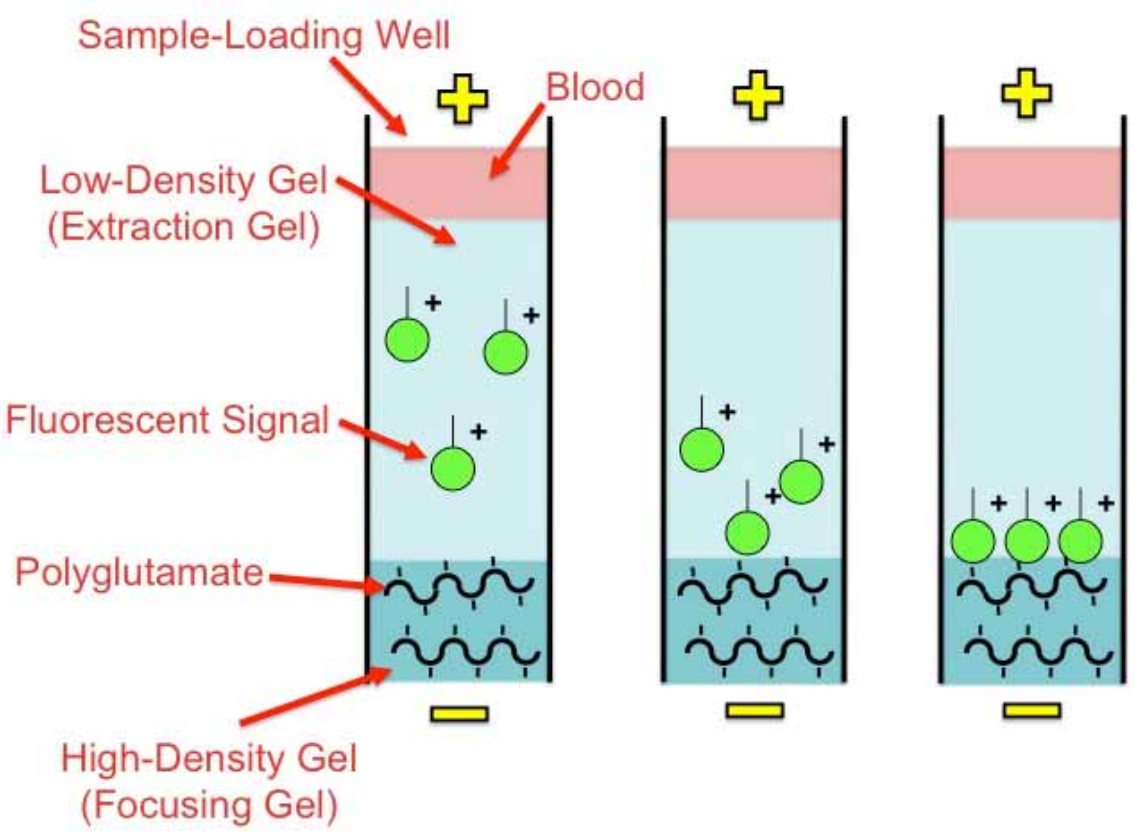

Figure 3.2: Mechanism for polyanionic focusing gel electrophoresis. The fluorescent signal is first separated from whole blood into a relatively low density (high porosity) "extraction gel." After it migrates through this gel and reaches the polyanion-doped higher density (low porosity) "focusing gel," there will be an electrostatic interaction between the positively charged signal fragment and the negatively charged dopant (polyglutamate). This interaction slows the migration of the signal fragment, which allows it to focus and thus improve the overall protease detection limit.

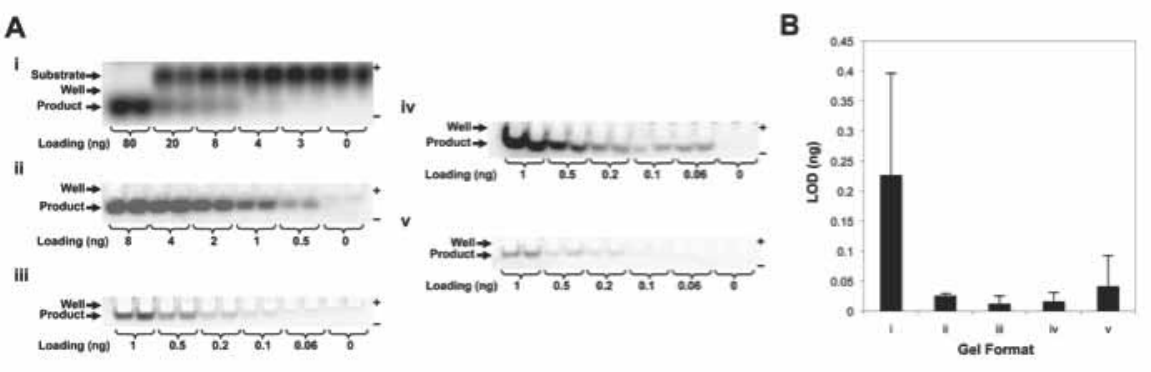

Figure 3.3: Detection of trypsin in 1X PBS in various agarose and polyacrylamide gel formats. In each case, 0.5 mg/mL of the substrate, Ac-N-DGDAGRAGAGK(ε-Bodipy FL)-NH_2, was reacted for 1 hour with several different concentrations of trypsin in 1X PBS and then loaded into a gel at 6 μL/well. A portion of this data was presented previously in [162]. (A) Shows the gel electrophoresis patterns for the following gel formats: (i) 1-cm thick horizontal 4% agarose gel and 1-mm thick vertical (ii) 20% (iii) 25% (iv) 35% and (v) 50% polyacrylamide gels. The amount of enzyme loaded into the gels is designated beneath the gel. The '+' and '-' denote the respective positions of the anode and cathode and the arrows designate the positions of the sample loading well, the intact substrate band, and the cleavage product band (the signal). Negatively charged substrate migrates toward the anode, while the fluorescent cleavage product migrates toward the cathode. (B) Shows the corresponding limits-of-detection for gel formats (i)-(v).

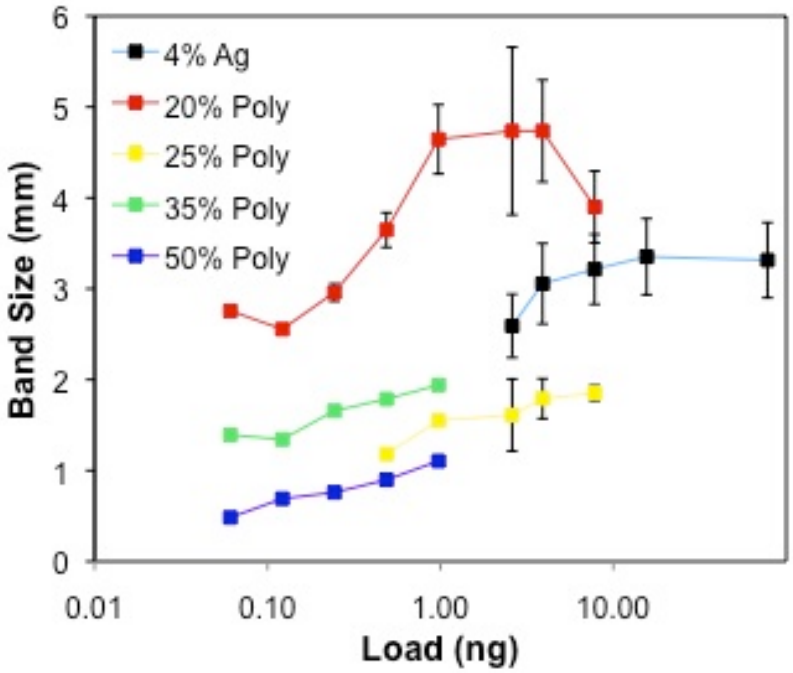

Figure 3.4: Fluorescent cleavage fragment band size in different gel types. The bands have different sizes depending on the amount of enzyme loaded into the gel, with larger bands produced by higher enzyme loading. As the gel density is increased, the bands become smaller due to the increased concentration of the cleavage fragment in lower porosity gels. This effect is limited beyond 25% (w/v) concentrations of acrylamide monomer (acrylamide plus bis-acrylamide cross-linker) because the gel's pore size asymptotically decreases (with increasing acrylamide concentrations) and provides negligible improvement in concentration of the cleavage fragment.

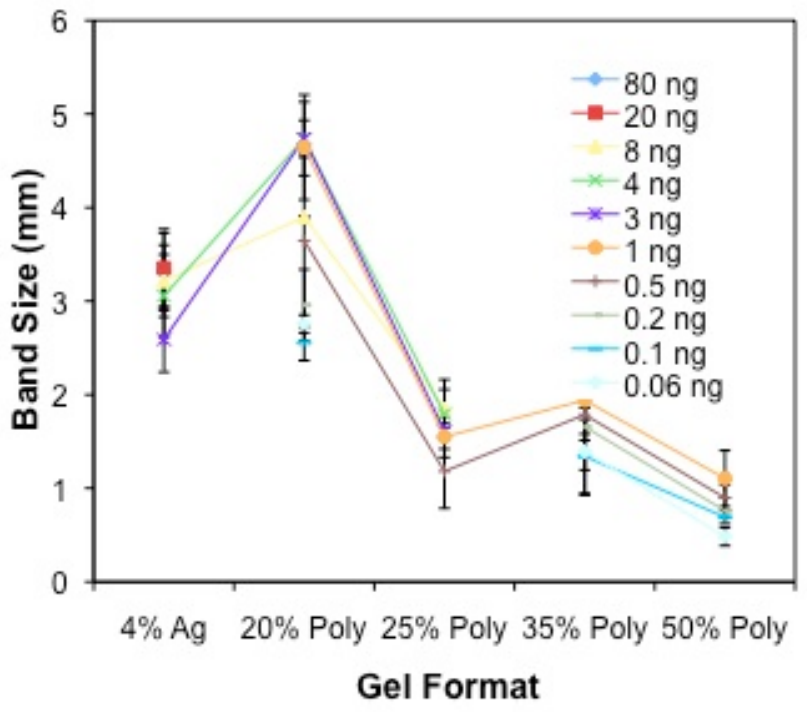

Figure 3.5: Fluorescent cleavage fragment band size in different gel types (alternate plot). Re-examining the data presented in Figure 3.4, it is evident that decreasing the gel density from that of a 4% agarose gel to that of a 25% polyacrylamide gel results in a decrease in the size of the cleavage fragment band by approximately two-fold. Further increases in gel density provide negligible improvement in band size.

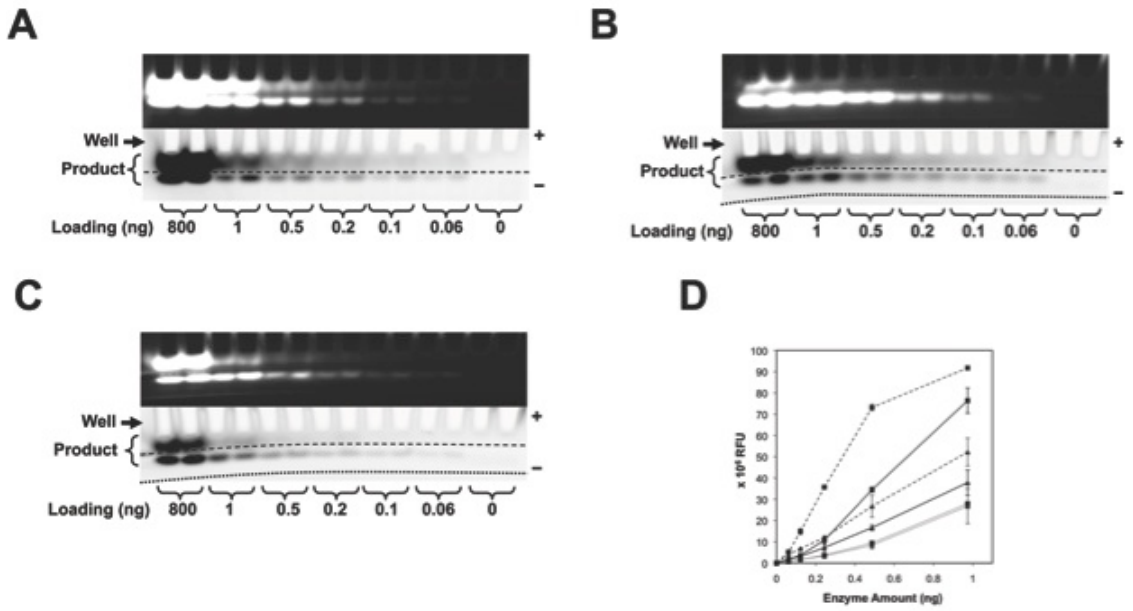

Figure 3.6: Detection of trypsin in 1X PBS with nonionic and polyanionic focusing gels. Following reactions as described in Figure 3.2, the reactions are electrophoresed across 12% polyacrylamide extraction gels and then against several types of focusing gels. (A)-(C) Shows photos (upper image) and quantitative gel scans (lower image) for the electrophoresis patterns for the following types of dopings in a 25% polyacrylamide focusing gel: (A) undoped (B) doped with 0.5% (w/v) PG1 and (C) doped with 0.5% (w/v) PG2. Dashed and dotted lines denote the bottom boundaries of the extraction and focusing gels, respectively. (D) Enzyme activity standard curves corresponding to gels in (A)-(C), with the signal reported in millions of relative fluorescence units (RFU). Solid, dashed, and dotted lines correspond to curves for undoped, PG1-doped, and PG2-doped focusing gels. Solid squares and solid triangles correspond to different repeats of the same experiment.

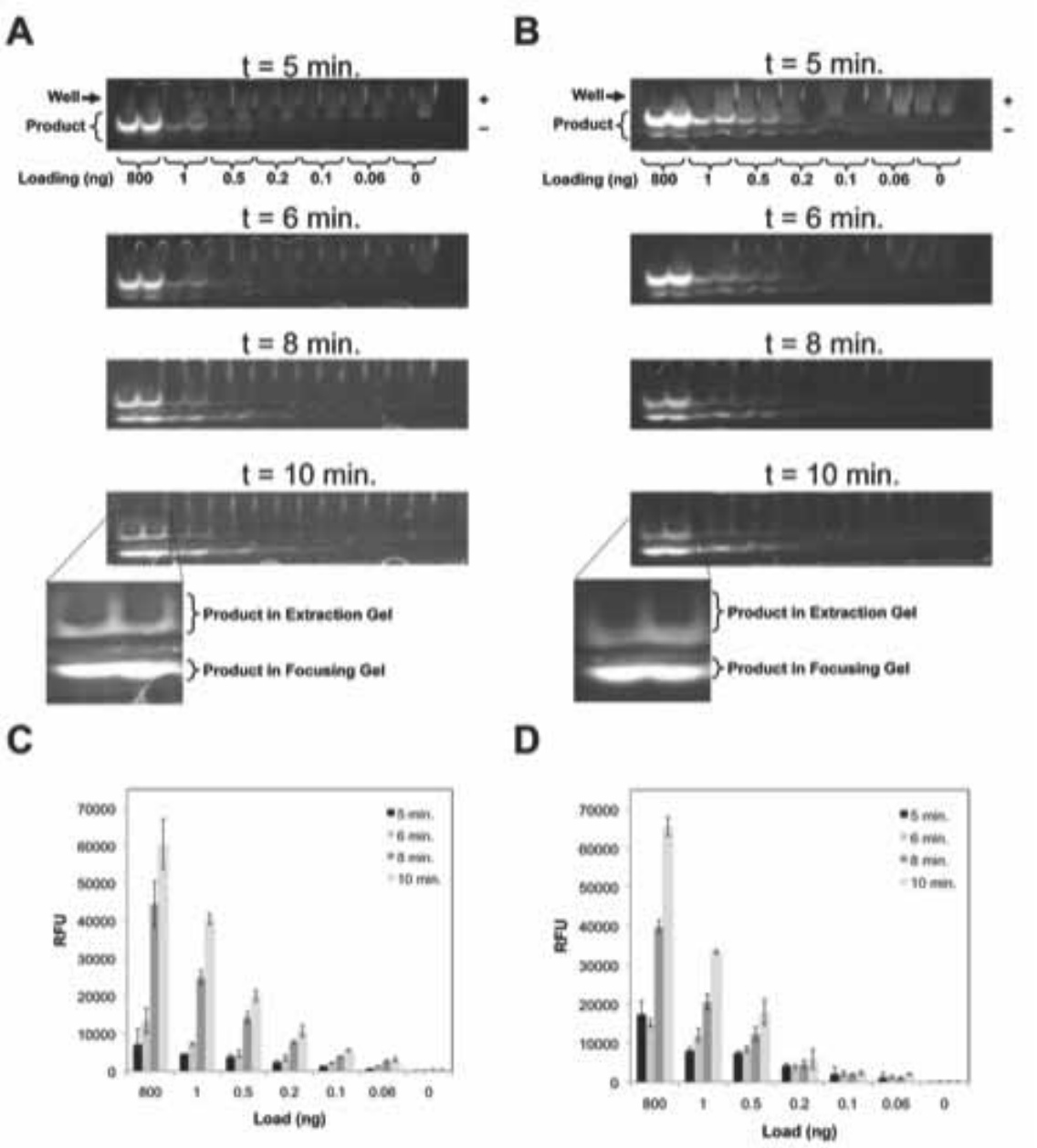

Figure 3.7: Time course of enhancement of signal for trypsin assay in HMW DNA-doped and PG1-doped focusing gels. Following reactions as described in Figure 3.2, the reactions are electrophoresed (at 6 μL/well loading) across a 12% polyacrylamide extraction gel and against a 25% polyacrylamide focusing gel doped with either 0.5% (w/v) sheared HMW DNA or 1% (w/v) PG1. (A)-(B) Shows gel electrophoresis patterns at time = 5 min., 6 min., 8 min., and 10 min. for a (A) 0.5% (w/v) HMW DNA-doped focusing gel and a (B) 1% (w/v) PG1-doped focusing gel. (C)-(D) Shows the change of RFU over time for different amounts of enzyme loaded into the gel for the respective gels shown in (A) and (B). Fluorescence is integrated over product within the focusing gel.

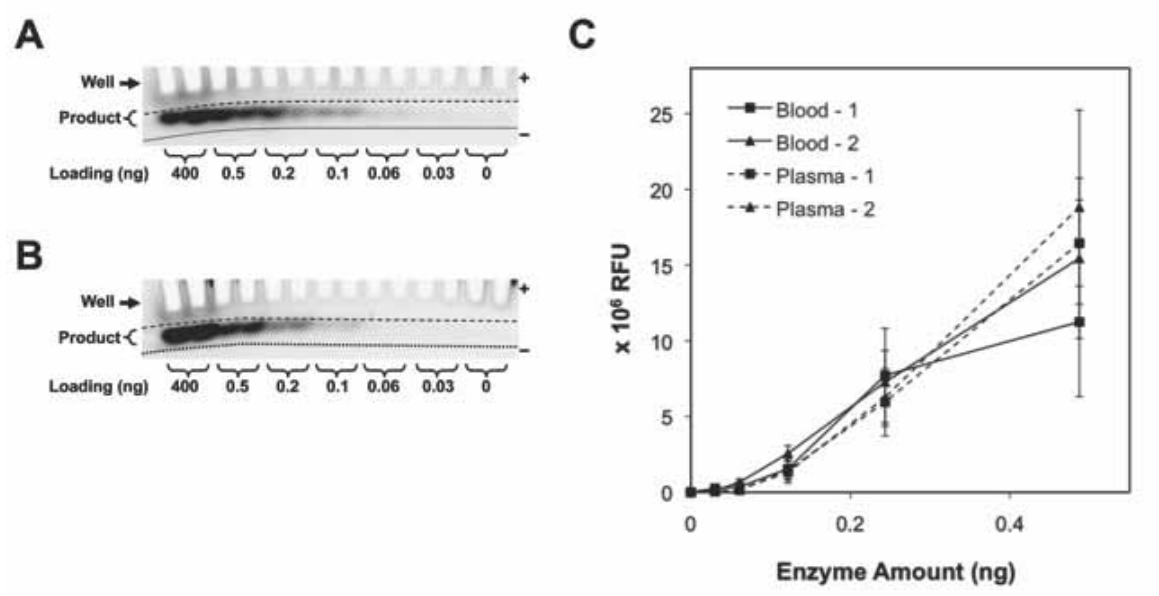

Figure 3.8: Detection of trypsin in human whole blood and plasma, using a PG1 focusing gel. Following 1X PBS reactions as described in Figure 3.2, the reaction mixtures are mixed 1:1 with protease-inhibitor treated blood or plasma immediately prior to electrophoresis. (A)-(B) Shows PG1 focusing gel electrophoresis patterns generated for detection of cleavage product generated from various levels of enzyme in (A) whole human blood and in (B) human plasma. (C) Shows enzyme activity standard curves corresponding to gels in (A)-(B), with the signal reported in millions of RFU. Solid and dashed lines correspond to curves for whole blood and plasma, respectively. Solid squares and solid triangles correspond to different repeats of the same experiment.

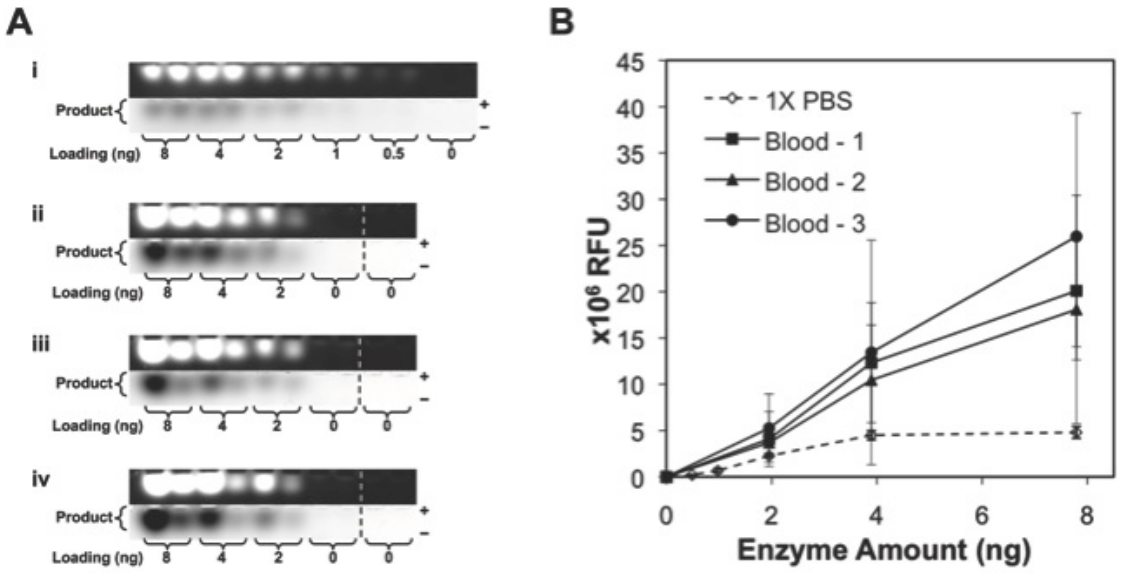

Figure 3.9: Detection of trypsin activity spiked into unseparated human whole blood samples. In each case (Gels i to iv), either 1X PBS (Gel i) or one of three human whole blood samples (Gels ii to iv) is mixed 1:1 with 1 mg/mL of trypsin substrate (or 1X PBS as a "no substrate" control). Selected concentrations of trypsin were added into this mixture and allowed to react for 30 minutes. These reactions were then loaded into 1-mm thick 20% polyacrylamide gels at 6 μL/well. (A) Shows gels (upper images) and quantitative gel scans (lower images) of the electrophoresis patterns for reactions in 1X PBS (Gel i) and in human whole blood samples 1-3 (Gels ii to iv). In Gels ii to iv, the lanes to the right of dashed line are "no substrate" controls (loaded with blood half-diluted with 1X PBS). (B) Enzyme activity standard curves corresponding to gels in (A), with the signal reported in millions of relative fluorescence units (RFU). Solid and dashed lines correspond to curves for whole blood samples and for 1X PBS, respectively. Solid squares, triangles, and circles correspond to reactions with blood samples 1-3, respectively.

Table 3.1: Trypsin detection limits in various gel formats

Exp Type	LOD (ng)	LOD (nM)	LOD (ng/ml)
4% Agarose	0.2	2	40
20% Polyacrylamide	0.03	0.2	4
25% Polyacrylamide	0.01	0.08	2
35% Polyacrylamide	0.02	0.1	3
50% Polyacrylamide	0.04	0.3	7
12%-25% Stacking	0.005	0.04	0.8
PG1 Stacking	0.001	0.008	0.2
PG2 Stacking	0.004	0.03	0.6

Chapter 3, in part, is a reprint of the material as it appears in Electrophoresis: Lefkowitz RB, Schmid-Schönbein GW, and Heller MJ, “Whole Blood Assay for Trypsin Activity Using Polyanionic Focusing Gel Electrophoresis,” *Electrophoresis* 2010, *31*(*14*), 2442-2451 (publisher Wiley-Blackwell). The dissertation author was the primary investigator and author of this paper.

Chapter 4:

Substrates for Elastase, Chymotrypsin, MMP-2, and MMP-9

4.1 Introduction

In Chapters 2 and 3, two charge-changing substrates were developed: (1) a common substrate for both α-chymotrypsin and trypsin and (2) a trypsin-specific substrate. In this chapter, this progress is extended by the development of new charge-changing substrates for the whole blood detection of elastase, chymotrypsin, MMP-2 and MMP-9 activity. This demonstrates the broad applicability of charge-changing substrates toward detection of enzymes that are potential diagnostic markers for many major diseases and medical conditions. For example, there are elevated levels of serum elastase in pancreatic cancer [4, 7, 8, 12, 14] and in pancreatitis [4, 8, 28]. In fact, several studies that have explored serum elastase as a potential early stage marker for pancreatic cancer [7, 12], which has a very poor prognosis and high mortality rate. Elevated levels of serum chymotrypsin are observed in acute pancreatitis and in renal failure [27]. MMP-2 and -9 levels are elevated in cancer [10], diabetes [19, 20], hypertension [24, 25], and in acute coronary syndrome [26]. Thus, the extension of the charge-changing substrate technique to these additional enzymes now creates new opportunities for the development of novel diagnostics and therapeutics beyond that created by the initial two substrates.

In particular, the ability to detect multiple enzymes is important for a very good reason: clinical specificity has typically been poor when using an individual protease marker. Many of the studies above [4, 7, 8, 10, 12, 14, 19, 20, 24-28] explored the utility of individual proteolytic enzymes in diagnosing various diseases. Unfortunately, there is often great overlap in the distribution of the levels (or activities) in the healthy and diseased patient populations. This limits the usefulness of a single protease marker in diagnostics because of low sensitivity, low specificity, or both. For example, in studies measuring elevated levels of MMP-2 and MMP-9 in patients with type 1 and type 2 diabetes, there was a strong overlap in the distributions of the levels found in healthy and diabetic populations [19, 20]. Thus, diagnosis of diabetes using the levels of MMP-2 or MMP-9 alone would not be accurate enough because of poor sensitivity and/or specificity. This issue can be overcome through multiplex detection. For example, the diagnostic accuracy for the diagnosis of pancreatic cancer was improved after employing a multivariate analysis of 23 serum markers (two of which were the proteases, trypsin and elastase) [13].

Using the new substrates developed in this chapter, very low LODs were achieved. In initial studies in 1X PBS, the LOD ranged from 1-40 pg (for 6 μL sample, 0.2-6 ng/mL) after only a one-hour reaction of enzyme and substrate. In a subsequent experiment measuring spiked protease in whole blood (with endogenous protease present), detection limits ranged from 100-200 ng/mL after a 1-hour reaction. Thus, these new substrates demonstrate broad applicability toward clinically relevant detection of multiple disease-relevant proteases.

4.2 Materials and Methods

4.2.1 Materials

The following peptide substrate sequences (S1-S5) were synthesized by Aapptec (Louisville, KY, USA): Ac-N-DGDAGRAGAGK-NH_2 (S1), Ac-N-DAGSVAGAGK-NH_2 (S2), Ac-N-DGDAAYAAYAGAG-diamino ethyl-NH_3 (S3), Ac-N-GDPVGLTAGAGK-NH_2 (S4), and Ac-N-GDLAAITAAGAGK-NH_2 (S5). Substrates S1, S2, S4, and S5 were subsequently labeled on the lysine residue's epsilon amine group with Bodipy FL-SE (BFL) (Invitrogen, Carlsbad, CA, USA) following the manufacturer's standard labeling protocol (see [162] or Chapter 2.2.1 for details). Substrate S3 was labeled with BFL at the terminal amine of the diamino ethyl group using this same protocol. The resulting conjugates for S1-S5 form fluorescence-tagged substrates, respectively, for trypsin (S1), elastase (S2), chymotrypsin (S3), both MMP-2 and MMP-9 (S4), and for both MMP-2 and elastase (S5). TPCK (N-tosyl-L-phenylalanyl chloromethyl ketone)-treated bovine pancreatic trypsin (T8802); porcine pancreatic elastase (E0258); bovine pancreatic α-chymotrypsin (CHY5S); recombinant human MMP-2 (M9070); recombinant human MMP-9 (M8945); 20,500 MW poly-L-glutamic acid (PG, P4761); high-resolution agarose (A4718); acrylamide (A3553); tris-borate EDTA buffer (TBE, T3913); Brij 35 (B4184); and α_1-antitrypsin (A9024) were obtained from Sigma-Aldrich (St. Louis, MO, USA). N,N'-methylenebisacrylamide (Bis, 2610-OP) and glycerol (GX0185-6) were obtained from EMD Chemicals (Gibbstown, NJ, USA). Ammonium persulfate (APS, BP179), N,N,N',N'-tetramethylethylenediamine

(TEMED, BP150), tris base (BP152), calcium chloride dihydrate (C79), and hydrogen chloride (A-144S), were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Sodium chloride (42429) was obtained from Acros Organics (Geel, Belgium). Novex pre-cast 1-mm thick 20%T 2.6%C polyacrylamide TBE gels & Novex gel cassettes (for casting custom 1-mm thick gels) were both obtained from Invitrogen. Phosphate buffered saline (PBS, 2810305) was obtained from MP Biomedicals (Solon, OH, USA). Peripheral blood was collected from volunteers into vacutainers containing heparin anticoagulant (BD Biosciences, San Jose, CA, USA). Plasma was obtained from these blood samples by collecting the supernatant after centrifugation at 1600g for 15 minutes.

4.2.2 Substrate Specificity

Enzyme stock solutions were prepared at 1.2 μM concentrations: bovine pancreatic trypsin (MW 23.8 kDa) and α-chymotrypsin (MW 25 kDa) in 1 mM HCl; porcine pancreatic elastase (MW 25.9 kDa) in 100 mM Tris-HCl (pH 8.0); and MMP-2 (MW 71 kDa) and MMP-9 (MW 77 kDa) in TCNB buffer (50 mM Tris (pH 7.5) with 10 mM $CaCl_2$, 150 mM NaCl, and 0.05% Brij 35). Five different solutions of 0.5 mg/mL of substrates S1-S5 (respective MW: 1293.1 (S1), 1150.9 (S2), 1536.0 (S3), 1361.2 (S4), 1434.2 (S5)) were prepared in 1X PBS (pH 7.8), with 0.6 mM $CaCl_2$. In a sixth solution, a solution of 0.5 mg/mL of substrate S5 was prepared in 1X PBS (pH 7.8), with 0.6 mM $CaCl_2$ and 400 nM α_1-antitrypsin, in order to test the modified specificity of this substrate in the presence of a serine protease inhibitor. Each of these 6 substrate solutions was then cross-reacted, in 6 sets of 6 individual reaction tubes, with each of the 5 enzymes or

with TCNB buffer (negative control) by spiking (1 μL volume) into the 0.5 mg/mL (14.4 μL) substrate solution. The final substrate and enzyme concentrations were approximately 0.5 mg/mL and 80 nM, respectively. If α_1-antitrypsin was present, the final concentration was 400 nM. After the reactions proceeded for 1 hour, 6 μL aliquots were loaded directly into the center of 7.5x10x1 cm 4% high-resolution agarose gels in 0.5X TBE (44.5 mM Tris-Borate, 1 mM EDTA, pH 8.0) and electrophoresed at 80V for 30 minutes. After electrophoresis, the gels were imaged directly by a BioDoc-It System with a Model M-26 transilluminator (UVP, Upland, CA, USA) at an excitation of 302 nm and emission of 500-580 nm. Gels were then quantified with a Storm 840 gel scanner (Molecular Dynamics, Sunnyvale, CA, USA) (fluorescence mode, high sensitivity, 100 μm pixel size, 1000V photomultiplier tube) with a 450 nm excitation filter and a 520 nm long-pass emission filter. The fluorescent signal was obtained using ImageQuant v5.2 by integrating the fluorescence over the entire cleavage product band and then subtracting the background fluorescence from the negative control.

4.2.3 Detection in 1X PBS using 25% Polyacrylamide Gels

Various concentrations of elastase, α-chymotrypsin , MMP-2, and MMP-9 were prepared in the following diluents: elastase in 100 mM Tris-HCl (pH 8.0); α-chymotrypsin in 1 mM HCl; and MMP-2 and MMP-9 in TCNB buffer. Solutions of 0.5 mg/mL of substrates S2- S4 were then prepared in 1X PBS (pH 7.8), with 0.6 mM $CaCl_2$ present only for α-chymotrypsin reactions. Different concentrations (1 μL volume) of a protease (or its diluent for a negative control) were spiked into the substrate solution

(14.4 μL), with each of the proteases reacted with the following substrates: elastase with S2, α-chymotrypsin with S3, MMP-2 with S4, and MMP-9 with S5. In this reaction mixture, the final concentration of substrate was 0.5 mg/mL. The final concentrations of enzyme were: 10, 6, 3, 2, 0.8, 0.4, and 0 nM for elastase and α-chymotrypsin; 3, 1, 0.7, 0.4, 0.2, 0.09, and 0 nM for MMP-2; and 3, 1, 0.7, 0.3, 0.2, 0.08, and 0 nM for MMP-9. After the reaction proceeded for 1 hour, aliquots of 6 μL of the reaction mixtures were mixed with 1 μL 35% glycerol (for a final concentration of 5% glycerol) and loaded directly into 8x8x0.1 cm custom-made vertical 25%T 6%C polyacrylamide gels that were cast into Novex gel cassettes. The samples were then electrophoresed at 500V for 10 minutes in 0.5X TBE running buffer. After electrophoresis, imaging and quantification were performed as described in Section 4.2.2.

4.2.4 Detection in 1X PBS using Focusing Gels

Polyanionic focusing gels consisted of three regions: a lower "filler" gel, an intermediate "focusing" gel doped with poly-L-glutamic acid (PG), and an upper "extraction" gel. The three gel regions were subsequently cast in 0.5X TBE buffer into a Novex gel cassette. The lower gel, cast first, was an 8x6.1x0.1 cm 12%T 6%C polyacrylamide gel that was allowed to polymerize for 1 hour. The intermediate gel, cast second, was an 8x0.5x0.1 cm 25%T 8%C polyacrylamide gel doped with 0.5% (w/v) PG (20.5K MW poly-L-glutamic acid). Polymerization of this gel proceeded for 0.5 hour. The uppermost gel, cast last, was an 8x0.3x0.1 cm 12%T 6%C polyacrylamide gel that was cast and allowed to polymerize for 0.5 hours. Following polymerization, a solution

of 0.5 mg/mL (14.4 μL) substrate (one of substrates S1-S5) was reacted with various concentrations (1 μL) of protease (or diluent for the negative control) in 1X PBS (pH 7.8) for 1 hour as described in Section 4.2.3, with protease reactions occurring with the following substrates: elastase with S2, α-chymotrypsin with S3, and MMP-2 and -9 with S4. The final concentration of substrate was 0.5 mg/mL. The final concentrations of enzyme were: 5000, 6, 3, 2, 0.8, 0.4, and 0 nM for elastase and α-chymotrypsin; 50, 1, 0.7, 0.4, 0.2, 0.09, and 0 nM for MMP-2; and 40, 1, 0.7, 0.3, 0.2, 0.08, and 0 nM for MMP-9. The highest concentration (5000 nM for elastase and α-chymotrypsin, 50 nM for MMP-2, and 40 nM for MMP-9) was tested simply to produce enough fluorescent signal such that the progress of the electrophoresis could be tracked by eye (either with excitation from ambient light or a handheld UV lamp). After completion of the reactions, aliquots of 6 μL of these mixtures were mixed with 1 μL 35% glycerol (for a final concentration of 5% glycerol) and loaded into these gels. After electrophoresis at 500V for 10 minutes, imaging and quantification were performed as described in Section 4.2.2, except that the fluorescence was integrated only for the portion of the cleavage product band that was within the focusing gel.

4.2.5 Detection of Spiked Protease in Whole Blood

Solutions of 1 mg/mL of the substrates S2-S4, in 1X PBS (pH 7.8), and selected dilutions of respective target proteases elastase, α-chymotrypsin, MMP-2, and MMP-9 (in their diluent), were prepared as described in Section 4.2.3. Samples (7 μL) of human heparinized whole blood obtained from healthy individuals were mixed 1:1 with 1

mg/mL (7 μL) substrate (or with 1X PBS (pH 7.8) as a "no substrate" control). Various concentrations (1 μL) of protease (or their diluent for the negative control) were spiked into the resulting mixture (14 μL) and allowed to react for 1 hour. The final substrate concentration was 0.5 mg/mL. For reactions with substrate present, the final enzyme concentrations tested were: 50, 30, 10, 6, 3 and 0 nM for elastase and α-chymotrypsin; 20, 10, 6, 3, 1, and 0 nM for MMP-2; and 20, 10, 5, 3, 1, and 0 nM for MMP-9. For the "no substrate" control, no protease was added. After completion of these 4 sets of reactions, aliquots of 6 μL of these mixtures were loaded into 4 separate 8x8x0.1 cm pre-cast Novex 20%T 2.6%C vertical polyacrylamide TBE gels. Following electrophoresis at 500V for 10 minutes, imaging, and quantification were then performed as described in Section 4.2.2.

4.3 Results and Discussion

Protease activity can be measured directly in *unprocessed* whole blood by designing charge-changing substrates that generate fluorescent positively charged cleavage fragments upon proteolysis [162, 165]. This eliminates the need for the sample preparation required by previous protease assays. Figure 4.1 demonstrates the basic steps for this whole blood assay, using, as an example, a charge-changing substrate for MMP-2 (substrate S5). The substrate is first mixed with a whole blood sample and allowed time to react with the target enzyme in the blood (Figure 4.1A). Prior to cleavage, the substrate, tagged with the fluorophore BFL, has a net negative charge of -1. Cleavage of the substrate by MMP-2 at the Ile-Thr bond produces a fluorescent cleavage product (the

signal) with a net charge of +1. The reaction mixture is then loaded into the sample well of an electrophoretic gel consisting primarily of two distinct regions, a low-density "extraction gel" (upper region) and a higher-density polyanion-doped "focusing gel" (lower region) (Figure 4.1B). Upon electrophoresis, the fluorescent positively charged cleavage fragment migrates into the extraction gel, where it is first separated from the predominantly negatively charged components of blood (cells, heme, plasma proteins, etc.) and from oppositely charged (uncleaved) substrate (Figure 4.1C). Subsequent electrophoresis of the cleavage fragment from the extraction gel into the focusing gel concentrates the fluorescent signal in order to achieve a lower detection limit (Figure 4.1D). This effect occurs because of the reduced mobility of the signal fragment in the denser gel and the electrostatic attraction of the positively charged cleavage fragment with the polyanion [165].

4.3.1 Substrate Specificity

In this chapter, four new substrates were developed: Ac-N-Asp-Ala-Gly-Ser-Val-Ala-Gly-Ala-Gly-Lys-(ε-BFL)-NH_2 (substrate S2), Ac-N-Asp-Gly-Asp-Ala-Ala-Tyr-Ala-Ala-Tyr-Ala-Gly-Ala-Gly-diamino ethyl-BFL (substrate S3), Ac-N-Gly-Asp-Pro-Val-Gly-Leu-Thr-Ala-Gly-Ala-Gly-Lys-(ε-BFL)-NH_2 (substrate S4), Ac-N-Gly-Asp-Leu-Ala-Ala-Ile-Thr-Ala-Ala-Gly-Ala-Gly-Lys-(ε-BFL)-NH_2 (substrate S5). Substrate S2 was developed for the detection of the pancreatic serine protease elastase, which can cleave substrates at peptide bonds on the carboxyl side of amino acid residues that have a small alkyl side chain. Thus, the amino acid sequence of substrate S2 has been designed to

include several Ala, Gly, and Val residues in order to promote cleavage by elastase (Table 4.1). Substrate S3 was developed as a substrate for the detection of the pancreatic serine protease α-chymotrypsin, which can cleavage peptide bonds on the carboxyl side of residues with large hydrophobic side chains. Thus, substrate S3 includes two Tyr residues in order to promote cleavage by α-chymotrypsin. Substrates S4 includes the sequence Pro-Val-Gly-Leu-Thr, which is a sequence that can be cleaved by MMP-9 at the Leu-Thr bond [166]. This sequence is part of the known MMP cleavage motif Pro-X-X-X_{Hy} (X is any residue and X_{Hy} is a hydrophobic residue), where cleavage occurs at the X-X_{Hy} bond. Thus, this collagen-like substrate can be cleaved by a number of MMPs and was incorporated into substrate S4 to promote cleavage by both MMP-2 and MMP-9. Substrate S5 includes the sequence Leu-Ala-Ala-Ile-Thr , which can be cleaved at the Ile-Thr bond by MMP-2 [167]. This sequence is known to be selective for MMP-2 versus MMP-9. Thus, while substrate S4 measures both MMP-2 and MMP-9 activity, substrate S5 is designed to specifically measure MMP-2 activity. Table 4.1 summarizes the sequences, protease targets, and charge-changes for substrates S1-S5 (substrate S1 from Chapter 2-3 ([162, 165]) is included for comparison).

Our first goal was to characterize the specificity of substrates S1-S5. For each substrate, 0.5 mg/mL of substrate was cross-reacted for 1 hour with 80 nM of (i) α-chymotrypsin, (ii) trypsin, (iii) elastase, (iv) MMP-2, (v) MMP-9, or (vi) buffer as a negative control. This enzyme concentration corresponds to 2 μg/mL for the serine proteases ((i)-(iii)) and to 6 μg/mL for the MMPs ((iv)-(v)). After completion of the 1-hour incubation, the reaction mixtures were loaded into 4% agarose gels and electrophoresed. The quantification of the resulting gel patterns for substrates S1-S5 are

shown in Figures 4.2A-4.2E, reporting fluorescent signal (F) as a percentage of maximum fluorescence (or activity) observed (i.e., $M = F/F_{max} \times 100\%$). The gel pattern for substrate S1 (Figure 4.2A) showed approximately an order of magnitude or more fluorescent signal for the intended target trypsin (ii) (M=100%) versus the other proteases (i, iii-v) and the negative control (vi). For substrate S2, the target protease elastase (iii) (M=100%) generated a larger signal than the other proteases (i-ii, iv-v) and the negative control (vi), with the highest cross-reactions observed with MMP-2 (iv) (M=56%) and MMP-9 (v) (M=47%) (Figure 4.2B). This result illustrates the challenge in making a specific substrate for elastase since this protease is relatively low in specificity, as compared to the other protease targets. Substrate S3 showed a strong preference for its intended target α-chymotrypsin (i) (M=100%) (Figure 4.2C). For this substrate, the only other enzyme that generated signal distinguishable from the negative control (vi) was elastase (iii) (M=25%). The highest fluorescent signals for substrate S4 were generated by reactions with intended targets MMP-2 (iv) (M=75%) and MMP-9 (v) (M=100%), with a 3-fold greater signal than for cross-reactions with the other proteases (i-iii) and the negative control (vi) (Figure 4.2D). This cross-reactivity with MMP-2 and -9 is consistent with the specificity predicted for Pro-Val-Gly-Leu-Thr, as described by Smith's group [166]. Finally, substrate S5 showed the greatest signal for reactions with MMP-2 (iv) (M=100%) and with elastase (iii) (M=75%) (Figure 4.2E). The expected strong specificity of substrate S5 for MMP-2 (iv) versus MMP-9 (v) cleavage is consistent with the specificity predicted for the sequence Leu-Ala-Ala-Ile-Thr [167]. Thus, these results show that the measured specificity of substrates S1-S5 is in good agreement with their designed protease specificity.

The cross-reactivity of substrates S3 and S5 by elastase (iii) is likely due to the small hydrophobic residues (e.g. Ala) in their amino acid sequence. Thus, a logical next step was to determine if protease inhibitors could be used in order to improve the substrate's specificity. In a 1-hour reaction, 0.5 mg/mL of substrate S5 was cross-reacted with 80 nM of each protease in the presence of the 400 nM α_1-antitrypsin, a serine protease inhibitor. As the results show in Figure 4.2F, MMP-2 (M=100%) generates approximately two-fold more activity than elastase (M=48%) in the presence of the serine protease inhibitor. Without the inhibitor, elastase cross-reactivity was higher (Figure 4.2E, M=75%). Thus, this simple experiment demonstrates that the specificity of substrate S5 can be improved with a serine protease inhibitor.

It should be noted that this estimation of specificity utilizes concentrations of protease that are higher than physiological concentrations of these proteases, which are generally in the range of 1-1000 ng/mL [4, 8, 10, 19, 20, 27], and this should be taken into consideration. For example, while substrate S4 can detect MMP-2 and elastase almost equally well (Figure 4.2D), measured mean levels of MMP-2 (1137.5 ng/mL) [19] are nearly 500-fold higher than for elastase (2.31 ng/mL) [4]. Thus, it is likely that there would be less cross-reaction of substrate S4 with elastase in physiological media. It should also be noted that specificity is often quantified by the determination of the Michaelis-Menten kinetics parameter k_{cat} and K_m, but these parameters are difficult to measure with limited supply of these substrates. The methods used in this chapter provide a rapid means of obtaining an estimate of the substrate specificity while using minimal amounts of substrate.

4.3.2 Detection in 1X PBS using 25% Polyacrylamide Gels

These next experiments determined the LOD of different proteases in 1X PBS buffer. The LOD for this assay depends on the type of electrophoretic gel format utilized, with 25% polyacrylamide and polyanionic focusing gels being the most sensitive formats developed thus far (see Chapters 2.3.5, 3.3.1, 3.3.2) [162, 165]. The classical gel format, a 1-mm thick 25%T 6%C vertical polyacrylamide gel, was the first to be examined. Various concentrations of elastase, α-chymotrypsin, MMP-2, and MMP-9 were reacted in 1X PBS with substrates S2, S3, S4, and S4, respectively. Reaction aliquots (6 μL) were then loaded into the gels and electrophoresed for 10 minutes at 500V. After electrophoresis, the fluorescent intensity of the cleavage product bands was integrated in each lane. Following IUPAC standards [157], the LOD of spiked enzyme was determined by the slope of the detection curve (m) and by the standard deviation of the negative control (s_b) by using the formula $LOD=3s_b/m$. Figures 4.3A-4.3D show the typical results for the detection of spiked elastase, α-chymotrypsin, MMP-2, and MMP-9, respectively, showing both the quantitative fluorescent gel scan (lower image) and a photo obtained from an imaging system (upper image). In each gel, each pair of lanes was loaded with a reaction mixture of substrate and a different concentration of enzyme, progressing from the highest concentration on the left-hand side of the gel to the negative control (no enzyme added) on the right-hand side. For the negative control, minimal positively charged fragments (likely substrate impurity) were detected since most of the fluorescence migrated toward the anode (substrate is negatively charged). As the amount of enzyme reacting with the substrate is increased (moving leftward), more substrate is

converted to product, resulting in an increasing amount of fluorescence migrating toward the cathode. The corresponding standard curves are shown in Figure 4.3E. The LOD (mean of 2 gel repeats) for each enzyme was estimated to be 0.01, 0.03, 0.005, and 0.002 ng for elastase, α-chymotrypsin, MMP-2, and MMP-9, respectively. This corresponds to concentrations of 2, 4, 0.5, and 0.4 ng/mL, respectively (for a 6 μL sample loading). By comparison, mean serum levels for elastase and α-chymotrypsin have been estimated, by radioimmunoassay, to be 2.31 ng/mL [4] and 37.5 ng/mL [27], respectively. For MMP-2 and -9, mean plasma levels have been estimated by a two-site ELISA approach to be 1137.5 ng/mL and 55.0 ng/mL [19]. Thus, detection using 25% polyacrylamide gels resulted in LODs that were approximately 1.2-fold, 9.4-fold, 2300-fold, and 140-fold better than established reference levels. The LOD for elastase is closer to its reference levels than the other three target proteases because this enzyme is present at the lowest concentrations. However, for α-chymotrypsin, MMP-2, and MMP-9, these results demonstrate detection of proteases levels that are 1-3 orders of magnitude lower than estimated reference levels.

4.3.3 Detection in 1X PBS using Focusing Gels

Experiments were next carried out to determine if even lower LODs could be achieved using the polyanionic focusing gel electrophoresis technique developed earlier (see Chapter 3) [165]. In this approach, the gel has three distinct regions. The uppermost 12% polyacrylamide “extraction” gel is for the initial separation of peptide cleavage fragment from blood. Below this gel section, there is a 25% polyacrylamide “focusing”

gel, doped with a 0.5% (w/v) 20,500 MW poly-L-glutamic acid (PG). This gel is for concentration of fluorescent signal. The lowermost region is a 12% polyacrylamide "filler" gel. As in the previous experiment of Figure 4.3, 0.5 mg/mL of substrates S2-S4 was reacted for 1 hour in 1X PBS with various concentrations of target protease. Samples were then loaded into the gel and electrophoresed at 500V. Electrophoresis was carried out for 10 minutes, which migrates most of the positively charged cleavage fragment into the focusing gel. Figures 4.4A-4.4D shows the detection of elastase with S2 (Figure 4.4A), α-chymotrypsin with S3 (Figure 4.4B), MMP-2 with S4 (Figure 4.4C), and MMP-9 with S4 (Figure 4.4D). Images are shown from both a gel scanner (lower image) and from an imaging system (upper image) in order to aid in identifying the boundaries between the different gel regions. The corresponding detection curves are shown in Figure 4.4E. The LOD of elastase, α-chymotrypsin, MMP-2, and MMP-9 (spiked into 1X PBS) was estimated to be: 0.04, 0.003, 0.003, and 0.001 ng, respectively (which corresponds to 6, 0.5, 0.2, and 0.2 ng/mL for 6 μL gel loading). For the last three enzymes, the LOD is 75-fold, 5700-fold, and 280-fold lower, respectively, than the estimated reference levels in plasma and serum. Thus, this demonstrates detection of ultra low concentrations for each of these enzymes. For elastase, the LOD did not improve and was still close to the estimated reference level.

Table 4.2 summarizes the LODs of these enzymes in both the 25% polyacrylamide gels and in the focusing gels (FG). For comparison, this table includes data from an earlier report (see Chapters 2-3) [162, 165] for the detection of trypsin with substrate S1. Chapter 3 showed that there was a 10-fold improvement using the polyanionic focusing gel versus a 25% polyacrylamide gel [165]. Similar to this result,

the detection of α-chymotrypsin, MMP-2, and MMP-9 was also improved in the focusing gel. Their LODs were approximately 8.2-fold, 1.7-fold, and 1.8-fold lower, respectively, than the results observed in the 25% polyacrylamide gel. For elastase detection, the LOD surprisingly increased 2.6-fold. We speculate that this variation in the improvement achieved by the focusing gel is likely due to a dependence on the size and hydrophobicity of the cleavage fragment, which varies for substrates S1-S4. Given a fixed gel pore size, smaller fragments will not be focused (concentrated) as much as larger fragments. Compared to the other substrates, the elastase substrate (S2) is expected to produce the smallest cleavage fragments (based on known elastase specificity, see Table 4.1). This may explain why the detection of elastase (by substrate S2) is not improved in the polyanion focusing gel. In addition, more hydrophobic fluorescent cleavage fragments may not interact as strongly with the polyanionic dopant in the focusing gel. This counters the concentration effect of the focusing gel since it is the electrostatic interaction between the polyanion and cleavage fragment that serves to retard migration of the cleavage fragment and aid in the concentration of the fragment. The cleavage fragment produced by substrate S4 (H_3N^+-Thr-Ala-Gly-Ala-Gly-Lys(ε-BFL)-NH_2) may be slightly more hydrophobic than the fragment produced by substrate S1 (H_3N^+-Ala-Gly-Ala-Gly-Lys(ε-BFL)-NH_2) due to the presence of an additional Thr residue. This is supported by an earlier study that modeled the hydrophobic and hydrophilic character of different amino acids within a protein. This study predicted that Thr would make an overall hydrophobic contribution even though it is polar residue [168].

4.3.4 Detection of Spiked Protease in Whole Blood

In our final experiment, our aim was to estimate the LOD for protease spiked directly into whole blood. This detection occurs in the presence of endogenous protease, protease inhibitors, and co-factors that are present in whole blood. Thus, it is important to note that there are three major differences between protease detection in buffer (1X PBS) and in a whole blood sample. First, there are protease inhibitors (e.g. α_1-antitrypsin and α_2-macroglobulin) in the blood that can block protease activity, lowering the detection signal. Second, there are endogenous proteases present in the blood that could potentially cleave the substrates, resulting in additional cleavage beyond that due to spiked enzyme. This would result in an increase in detection signal. These enzymes may be the same protease as the spiked enzyme (e.g. endogenous MMP-2 or -9 detected by substrate S4), or they may be proteases that have similar specificity (e.g. other MMPs). Finally, there are co-factors (e.g. zinc, calcium) that are present in the blood that can aid in the function and activity of the proteases. Thus, for the same concentration of enzyme, there may be a different signal generated from a protease in blood than in buffer, as observed in our studies (see Chapters 2-3) [162, 165].

Given the expected background signal from the known reference levels of elastase [4], α-chymotrypsin [27], and of MMP-2 and -9 [19], and given the LODs that were achieved in previously explored gel formats [162, 165], it was sufficient to estimate the LOD of spiked enzyme using pre-cast 20% polyacrylamide gels (rather than more sensitive 25% polyacrylamide gels or polyanion focusing gels). After a 1:1 mixing of whole blood samples and 1 mg/mL substrate, various concentrations of protease were

spiked into these mixtures and allowed to react with substrate for 1 hour. In an additional "no substrate" control, blood was also mixed 1:1 with 1X PBS to see the contribution of blood itself to the detection signal. Each experiment was repeated twice with 2 different gels, using blood samples from 2 different individuals. Figures 4.5A-4.5D show typical gel patterns for the detection of elastase with S2 (Figure 4.5A), α-chymotrypsin with S3 (Figure 4.5B), and MMP-2 and -9 with S4 (Figures 4.5C-4.5D, respectively). The "no substrate" control (see pair of lanes to the right of the dashed lines), show the fluorescent signal generated from blood in the absence of any addition of substrate or spiked enzyme. For these controls, no discernable fluorescence was detected, showing that fluorescence signal is observed only upon addition of substrate and upon cleavage by either endogenous protease or spiked protease. Upon addition of substrate, but without the spiking of enzyme, fluorescent cleavage fragment is produced with all 3 substrates (S2-S4) (see pair of lanes immediately to the left of the dashed lines). On average (2 different blood samples), the observed background signal was highest for substrate S4 (Figures 4.5C-4.5D) and lowest for substrates S2 and S3 (Figures 4.5A-4.5B). This is consistent with the known reference levels of these enzymes, which, for example, show the highest concentration for MMP-9 [19]. This is also consistent with the LODs of these substrates, since the LODs of substrate S4 (for MMP-2 and -9) are much lower than the LODs of substrates S2-S3 (for elastase and α-chymotrypsin) (Table 4.2). Moving further leftward in the 4 gels, Figures 4.5A-4.5D show the effect of spiking in 0.5 ng to 8 ng (3 nM to 50 nM) of enzyme for elastase and α-chymotrypsin and 0.6 ng to 10 ng (1 nM to 20 nM) for MMP-2 and -9. The corresponding detection curves are shown in Figure 4.5E. The fluorescence values after background subtraction and expression in relative

fluorescence units (RFU), show, for all 4 enzymes and for all concentrations tested, higher signal with spiked enzyme versus the negative control (no enzyme added).

The LOD of enzyme spiked into whole blood was estimated to be 1, 1, 0.7, and 0.7 ng for elastase, α-chymotrypsin, MMP-2, and MMP-9, respectively. For 6 μL sample loading, this corresponds to approximately 200, 200, 100, and 100 ng/mL, respectively, which are higher detection limits than observed for the detection in 1X PBS (Figures 4.3-4.4). There are at least three possible explanations for the higher LODs observed in the whole blood detection experiment of Figure 4.5. First, the 20% polyacrylamide gel format used does not achieve an LOD as low as in the 25% polyacrylamide gel and the polyanionic focusing gel formats [165]. Second, as evident in the literature [4, 19, 27], there is a higher background in whole blood due to endogenous levels of protease. This effect is not shown in the curves of Figure 4.5E due to the background subtraction (data is shown in RFU). As the background (b) and, consequently, the standard deviation of background (s_b), become higher, so does the LOD (recall, LOD = $3s_b/m$). Finally, it is possible that endogenous protease inhibitors block some of the activity of the spiked enzyme. Regardless, even though the LODs are increased in this experiment, they still compare well with the reference levels for these enzymes (2.31 ng/mL [4], 37.5 ng/mL [27], 1137.5 ng/mL [19], and 55.0 ng/mL [19], respectively). In the worst case, the LOD for elastase is approximately 100-fold higher than the reference levels. However, the LOD for α-chymotrypsin and MMP-9 are only 5-fold and 2-fold higher, respectively, than the reference levels. For the best case, achieved for MMP-2, the LOD is 9-fold lower than the estimate reference level. Given the close proximity of the LODs of these enzymes to the known reference levels, there are several simple ways of bridging the gap

toward clinically relevant detection. For example, the LOD can be improved through the use of more sensitive gel formats (25% polyacrylamide gels, polyanionic focusing gels). The LOD can also be very simply improved through longer reaction times (>30 minutes) and larger sample loading (>6μL). Thus, the results for the detection of different proteases spiked directly into whole blood confirm that it is feasible to detect low levels of protease activity directly from unprocessed whole blood samples.

4.4 Concluding Remarks

In this study, charge-changing substrates were designed and tested for the purpose of detecting the activities of elastase, α-chymotrypsin, MMP-2, and MMP-9 *directly* in whole blood. In this assay, charge-changing substrates are cleaved by the target enzyme to produce a positively charged cleavage fragment. This fragment is rapidly separated from whole blood components by electrophoresis, eliminating the need for sample preparation (see Chapters 2-3) [162, 165]. The LOD is further improved by subsequent electrophoresis into a polyanion-doped polyacrylamide gel. Thus far, we have demonstrated LODs in whole blood that are close to the established reference levels for these enzymes, after only 30 minutes of reaction time and using only 6 μL of sample. This shows that it is feasible to achieve rapid, *clinically* relevant detection of these proteases in minimal volumes of whole blood. Further improvement in the LOD can be obtained with the incorporation of thinner microgel formats and more sensitive fluorescent labeling (e.g. quantum dots, nanoparticles), which will also aid in lowering the required reaction time. Gel miniaturization (shorter gel lengths) and higher voltage

gradients can also speed up the electrophoresis time. With such improvements, we envision that the entire process could be performed as rapidly as several seconds to several minutes. As this chapter demonstrates, this simple assay is broadly applicable to the detection of many different proteases, having so far shown the whole blood detection of five different proteases. This technique can be further extended to the detection of other types of degradative enzymes such as nucleases, lipases, and amylases. The ability to rapidly measure clinically relevant levels of protease activity directly in whole blood, without any sample preparation, overcomes one of the major limitations in developing viable, accurate, and rapid POC tests.

4.5 Acknowledgements

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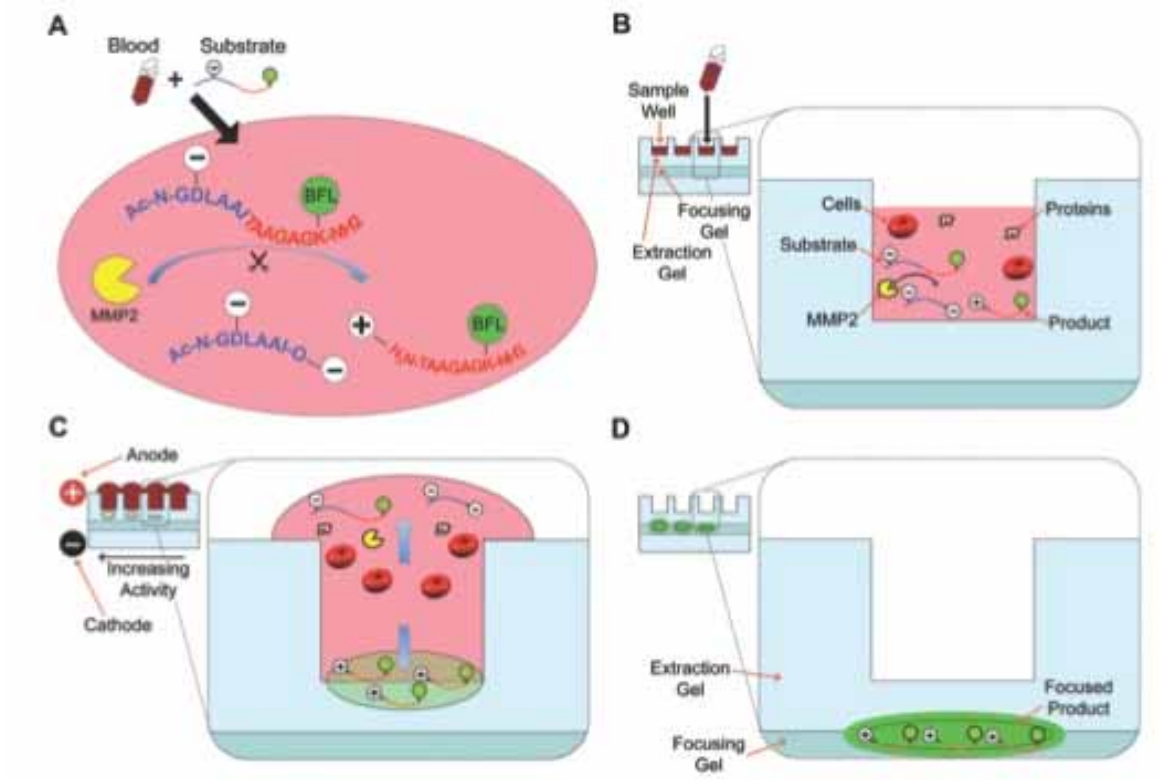

Figure 4.1: Schematic of a whole blood assay of MMP-2 activity that utilizes a charge-changing substrate. (A) In the first step of the assay, a fluorescence-labeled negatively charged substrate is added to a whole blood sample. Proteolysis of the substrate by its target enzyme MMP-2 generates a fluorescent positively charged cleavage product. (B) The reaction mixture is then loaded into the well of an electrophoretic gel consisting of two regions, an “extraction gel” for separation of the signal from whole blood and a “focusing gel” for subsequent concentration of the signal. (C) During electrophoresis, blood cells, plasma proteins, and negatively charged uncleaved substrate migrate toward the anode. The positively charged fluorescent signal migrates in the opposite direction into the extraction gel and is resolved from blood components. (D) Subsequent electrophoresis into the higher density, polyanion-doped focusing gel concentrates the fluorescent signal and facilitates lower detection limits.

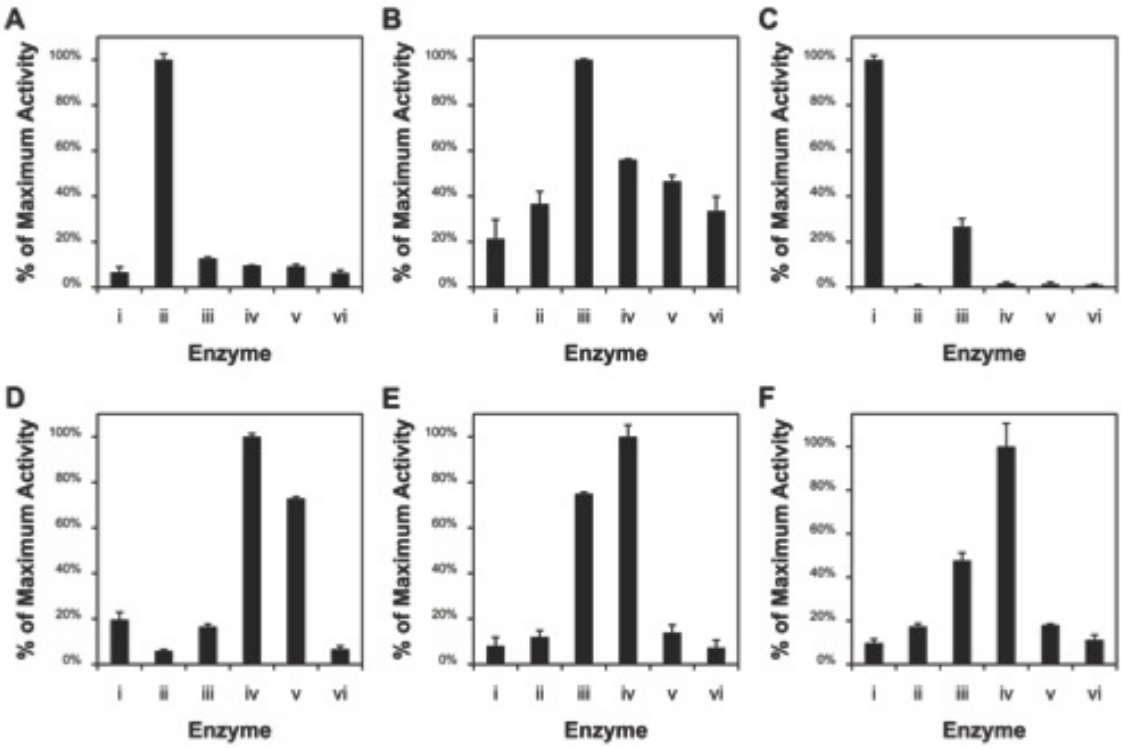

Figure 4.2: Specificity of substrates (A) S1, (B) S2, (C) S3, (D) S4, and (E) S5 with no protease inhibitors present. (F) Shows the altered specificity of S5 in the presence of 400 nM α_1-antitrypsin. In each case (A)-(F), fluorescent intensities were obtained after 1 hour reactions at 21ºC between 0.5 mg/mL of substrate and 80 nM of (i) α-chymotrypsin, (ii) trypsin, (iii) elastase, (iv) MMP-2, (v) MMP-9, or (vi) a negative control in 1X PBS. Next, samples were electrophoresed in 4% agarose gels (6 μL sample/well). Fluorescence values are reported as a % of maximum fluorescence observed.

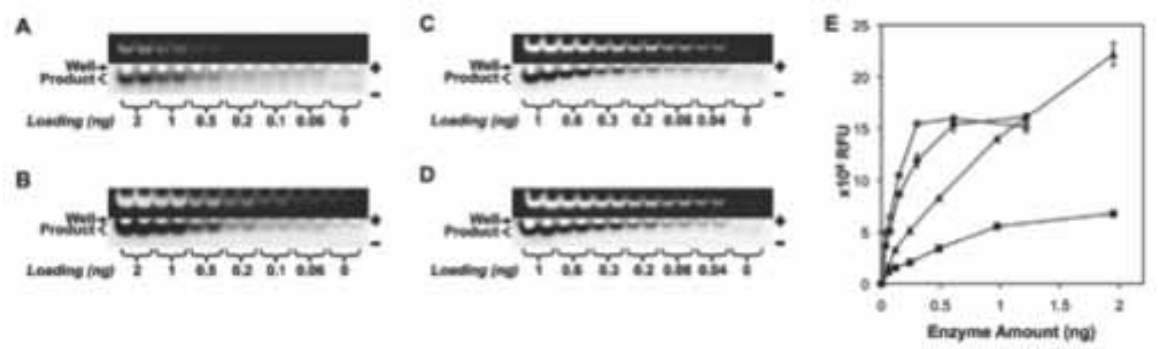

Figure 4.3: Detection of various proteases in 25% polyacrylamide gels. (A)-(D) Shows photos (upper image) and quantitative gel scans (lower image) for the electrophoresis patterns generated from detection of (A) elastase with substrate S2, (B) α-chymotrypsin with substrate S3, (C) MMP-2 with substrate S4, and (D) MMP-9 with substrate S4 in 1X PBS, using 25% polyacrylamide gels. In each case, 0.5 mg/mL of the substrate was reacted for 1 hour with several different concentrations of protease in 1X PBS and then loaded into a gel at 6 μL/well. The amount of enzyme loaded into the gels is designated beneath the gel. The '+' and '-' denote the respective positions of the anode and cathode and the arrows designate the positions of the sample loading well and the cleavage product band (the signal). Negatively charged substrate migrates toward the anode into running buffer, while the fluorescent cleavage product migrates into the gel toward the cathode. (E) Shows the corresponding detection curves from the gels of (A)-(D). Solid squares, solid triangles, solid circles, and empty circles correspond to gels of (A)-(D), respectively. Fluorescence is reported in millions of relative fluorescence units (RFU).

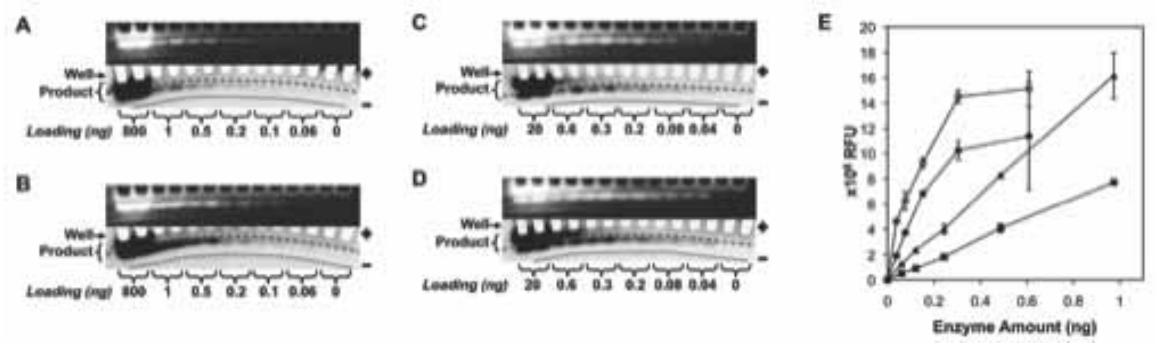

Figure 4.4: Detection of various proteases in PG focusing gels. Following reactions as described in Fig. 4.3, the reaction mixtures are electrophoresed across a 12% polyacrylamide extraction gel and then against a PG focusing gel. (A)-(D) Shows photos (upper image) and quantitative gel scans (lower image) for the electrophoresis patterns generated from detection of (A) elastase with S2, (B) α-chymotrypsin with S3, (C) MMP-2 with S4, and (D) MMP-9 with S4 in 1X PBS. Dashed and dotted lines denote the bottom boundaries of the extraction and focusing gels, respectively. (E) Shows the corresponding detection curves from the gels of (A)-(D). Solid squares, solid triangles, solid circles, and empty circles correspond to gels of (A)-(D), respectively.

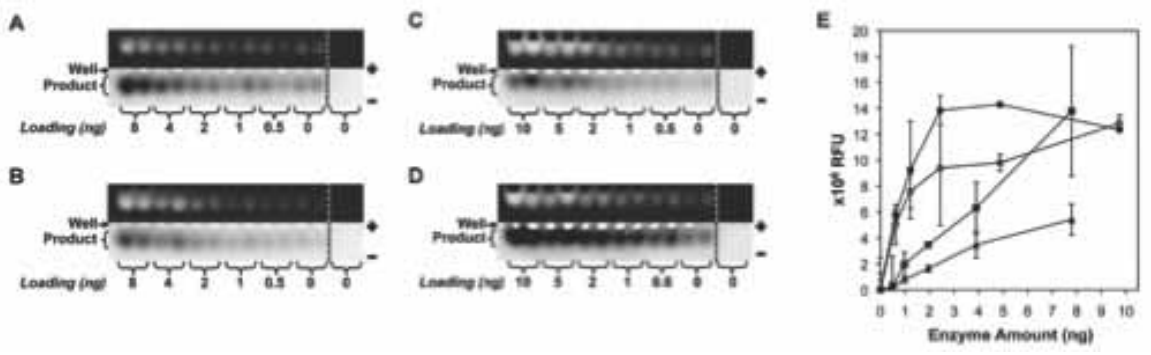

Figure 4.5: Detection of various proteases spiked into whole blood. (A)-(D) Shows photos (upper image) and quantitative gel scans (lower image) for the electrophoresis patterns generated from detection of (A) elastase with S2, (B) α-chymotrypsin with S3, (C) MMP-2 with S4, and (D) MMP-9 with S4. In each case (A)-(D), human whole blood is mixed 1:1 with 1 mg/mL of substrate, or 1X PBS as a "no substrate" control. Various concentrations of protease were then added into these mixtures and allowed to react for 1 hour. These reactions were then loaded into 1-mm thick 20% polyacrylamide gels at 6 μL/well. The "no substrate" controls are shown in the two lanes to the right of dashed line (loaded with blood half-diluted with 1X PBS). (E) Enzyme activity standard curves corresponding to gels in (A)-(D), with solid squares, solid triangles, solid circles, and empty circles corresponding to gels of (A)-(D), respectively.

Table 4.1: Sequences of S1-S5 and their cleavage sites

Peptide	Target Protease	Sequence and cleavage site	Charge	
			Substrate	*C-terminal Product*
S1	Trypsin	Ac-N-D⁻-G-D⁻-A-G-R⁺-A-G-A-G-K(ε-BFL)-NH_2	-1	+1
S2	Elastase	Ac-N-D⁻-A-G-S-V-A-G-A-G-K(ε-BFL)-NH_2	-1	+1
S3	Chymotrypsin	Ac-N-D⁻-G-D⁻-A-A-Y-A-A-Y-A-G-A-G-diamino ethyl-BFL	-2	+1
S4	MMP-2/9	Ac-N-G-D⁻-P-V-G-L-T-A-G-A-G-K(ε-BFL)-NH_2	-1	+1
S5	MMP-2	Ac-N-G-D⁻-L-A-A-I-T-A-A-G-A-G-K(ε-BFL)-NH_2	-1	+1

Table 4.2: Detection limits for substrates S1-S5 in multiple gel formats[a]

Peptide	Target Protease	ng		ng/ml		nM	
		25%	FG	25%	FG	25%	FG
S1	**Trypsin**	0.01	0.001	2	0.2	0.08	0.008
S2	**Elastase**	0.01	0.04	2	6	0.09	0.2
S3	**Chymotrypsin**	0.03	0.003	4	0.5	0.2	0.02
S4	**MMP-2**	0.005	0.003	0.5	0.2	0.009	0.004
S4	**MMP-9**	0.002	0.001	0.4	0.2	0.005	0.002

[a]Data for trypsin was reported previously [162, 165].

Chapter 4, in part, has been submitted for publication of the material as it may appear in Analytical Chemistry: Lefkowitz RB, Schmid-Schönbein GW, and Heller MJ, "Whole Blood Assay for Elastase, Chymotrypsin, MMP-2, and MMP-9 Activity," *Analytical Chemistry* (Submitted for Publication June 2010) (publisher American Chemical Society). The dissertation author was the primary investigator and author of this paper.

Chapter 5:

Measurement of Protease Activity in Disease

5.1 Introduction

To avoid the label “technology without an application, ” it is important that several applications were explored for this newly developed whole blood protease assay. Complete clinical trials can take years and millions of dollars and are beyond the scope of this dissertation. However, it is certainly feasible to demonstrate smaller scale proof-of-concept studies to show the value of this novel assay and to point to possible future applications. Toward this end, Chapter 5 discusses the application of charge-changing substrates to the measurement of protease activity in clinical samples for type II diabetes, physiological shock, and in pancreatic cancer.

In the first application that is explored, type II diabetes, elevated MMP levels have been observed using ELISA [19, 23]. Lee et al. measured the concentration levels of MMP-2, MMP-9, and TIMP-1 (a natural MMP inhibitor) in non-fasting lithium plasma samples from 80 normal controls and 80 type II diabetics [19]. In the normal controls, the measured levels were 1137.5 ± 299.2 ng/mL (mean ± s.d.), 55.0 ± 40.0 ng/mL, and 172.5 ± 184.1 ng/mL, respectively. In the diabetics, the measured levels were 1239.6 ± 309.0 ng/mL, 49.8 ± 46.2 ng/mL, and 362.1 ± 187.7 ng/mL, respectively. For MMP-2 and TIMP-1, they reported a significant ($p<0.05$) elevation using a Student’s *t*-test. In another study by Derosa et al., using fasting sodium heparin plasma samples from an even larger study population of 165 normal controls and 181 diabetics, they measured the protein

levels of MMP-2, MMP-9, TIMP-1, and TIMP-2 [23]. In the normal controls, the corresponding levels were 628.1 ± 267.4 ng/mL, 53.7 ± 16.0 ng/mL, 166.9 ± 56.4 ng/mL, and 80.1 ± 5.9 ng/mL, respectively. In the diabetics, the corresponding levels were 1351.3 ± 153.9 ng/mL, 521.7 ± 63.9 ng/mL, 510.7 ± 49.9 ng/mL, and 99.9 ± 3.5 ng/mL, respectively, with statistically significant ($p<0.0001$) elevations of all four markers. The differences between these two studies can possibly be attributed to 1) in-homogeneity due to limited size of study populations, 2) lack of control for differences in therapies (some may be treated with MMP inhibitors), 3) differences in fasting, and 4) differences in ethnic origin of the study population. As discussed in Chapter 1.5, there are also many ways that the type of sample preparation can affect both the activities and protein concentration levels of various MMPs in clinical samples [141-147, 149, 169]. Regardless, the studies, even though sometimes conflicting, together show elevated levels of MMPs in type II diabetics. Whether it is MMP-2 or MMP-9, or both, the charge-changing substrate developed in Chapter 4 (substrate S4) can detect elevated activity of either protease in whole blood.

This provides an exciting opportunity in developing novel therapeutics for type II diabetes. As discussed in Chapter 1.3, DeLano et al. showed, in spontaneously hypertensive rats using immunohistochemistry and in vivo microzymography, elevated levels of MMP-1 and -9 activity as well as a corresponding increase in insulin receptor cleavage [24]. They also observed elevated protease activity in SHR plasma using a fluorogenic substrate. Together, this evidence shows that elevated MMP activity leads to proteolytic cleavage of the insulin receptor and the subsequent development of insulin resistance seen in this rat strain. This same process could be a potential

pathophysiological mechanism that leads to type II diabetes. By detecting elevated MMP activity in diabetics, a logical possible treatment would be an MMP inhibitor. Thus, even though there is already a gold standard for diagnosing diabetes (glucose levels), the ability to rapidly measure this new diabetic marker in whole blood could be useful for making a decision to treat a patient with an MMP inhibitor.

Physiological shock is the second application that will be explored in this dissertation. As discussed in Chapter 1.2, physiological shock is a serious, life-threatening condition that has a high mortality rate (51.5%) and that can progresses to deadly multi-organ failure (MOF) within hours [34, 35]. In order to successfully intervene in this condition and prevent MOF, first responders and physicians need a rapid early-stage diagnostic test, which unfortunately does not currently exist. If any potential future diagnostic test requires sample preparation, it will be more time-consuming, more costly, and less accurate than a test that does not require sample preparation. Even more of an issue, if the test is too complex (because of sample preparation), it will need to be performed in a CLIA-approved central laboratory. For shock, this is simply too risky to the patient to lose precious time waiting for hours to days for a central laboratory to finish sample testing. A viable shock diagnostic requires the ability to rapidly measure early-stage markers *directly* in whole blood and at the point-of-care.

Proteases have been implicated early in the progression of shock. In shock, digestive enzymes (e.g. amylase, lipase, chymotrypsin, trypsin, elastase) synthesized in the pancreas as part of normal digestion find entry into the wall of the intestine where they can target a variety of autologous protein and lipid structures (Figure 1.1) [18, 37, 40, 41]. In this “auto-digestion hypothesis,” inflammatory mediators generated from this

activity can enter the circulation, cause systemic inflammation, and eventually cause MOF and death. Intraintestinal treatment with protease inhibitors has been shown to ameliorate inflammation and the progression of shock [170, 171]. It is possible that some of the digestive enzymes can themselves also escape from the intestine and into the circulation. Using a radioimmunoassay, Florholmen et al. measured elevated serum levels of serum cationic trypsin-like immunoreactivity in hemorrhagic and nortriptyline-induced shock (cardiogenic shock) [172]. Malinoski et al. showed that elevated serum amylase and lipase were predictive indicators for organ failure and death [173]. In an unpublished study by Dr. Alexander Penn and Professor Geert W. Schmid-Schönbein, elevated plasma levels of activity were observed for chymotrypsin, trypsin, and elastase using a chromogenic substrate (presented below in Figures 5.6 and 5.8). If we can detect these markers, directly in whole blood, it may become possible, for the first time, to develop an early-stage, rapid POC diagnostic for physiological shock.

The third and final disease application that was explored is pancreatic cancer. Pancreatic cancer has a poor prognosis. It is the fourth leading cause of cancer death and has one-year and five-year survival rates of 6% and 25%, respectively [174]. Broken down by stage of diagnosis, the 5-year survival rate is 22.5% when the tumor is confined to the pancreas, 8.8% when it has spread to regional lymph nodes, and 1.9% when it has reached the metastatic stage [175]. Thus, the best outcome for treatment of pancreatic cancer is obtained by early diagnosis. Unfortunately, there is no current method for early detection of pancreatic cancer. This disease is usually asymptomatic during early stages and is not diagnosed until later stages. Thus, it is very difficult to identify early stage markers. Tseng et al. have developed an orthotopic model for pancreatic cancer in an

incompetent murine host [176]. This model mimics the genetic mutations, histological appearance, and pattern of disease progression of human pancreatic cancer. Using genetic models that mimic human pancreatic cancer, it becomes feasible to search more effectively for early stage markers. As reviewed in Chapter 1.3, the pancreatic protease elastase has been repeatedly explored as a potential diagnostic marker in serum for pancreatic cancer [4, 7, 8, 12, 14]. Several studies have indicated its potential as an early stage marker [7, 12]. Unfortunately, during the time of those studies, they did not have a genetic mouse model to see if these markers are sensitive and specific for a sufficiently early stage (pre-stage 1). Furthermore, these studies are only measuring the levels of an individual marker, which is generally not effective since individual markers often lack sufficient sensitivity and specificity for diagnosis.

Using a genetic mouse model, we can now begin to search for early stage markers. The charge-changing substrate assay is an ideal choice for this task, as it can look for markers directly in unprocessed whole blood and make its determination more accurate. Since this assay is amenable to multiplexation, it can be used for screening for multiple markers simultaneously. Once accurate early-stage markers are discovered, this technology could then be used to develop a low cost and rapid point-of-care diagnostic. The combination of a genetic mouse model and the charge-changing substrate assay is a promising new direction for developing an early stage pancreatic cancer diagnostic.

5.2 Materials and Methods

5.2.1 Materials

The following peptide substrate sequences (S1-S4) were synthesized by Aapptec (Louisville, KY, USA): Ac-N-DGDAGRAGAGK-NH_2 (S1), Ac-N-DAGSVAGAGK-NH_2 (S2), Ac-N-DGDAAYAAYAGAG-diamino ethyl-NH_3 (S3), and Ac-N-GDPVGLTAGAGK-NH_2 (S4). Substrates S1, S2, and S4 were subsequently labeled on the lysine residue's epsilon amine group with Bodipy FL-SE (BFL) (Invitrogen, Carlsbad, CA, USA) following the manufacturer's standard labeling protocol (see Chapter 2.2.1 or [162] for details). Substrate S3 was labeled with BFL at the terminal amine of the diamino ethyl group using this same protocol. The resulting conjugates form fluorescence-tagged substrates, respectively, for trypsin (S1), elastase (S2), chymotrypsin (S3), and for both MMP-2 and MMP-9 (S4). TPCK (N-tosyl-L-phenylalanyl chloromethyl ketone)-treated bovine pancreatic trypsin (T8802); porcine pancreatic elastase (E0258); bovine pancreatic α-chymotrypsin (CHY5S); recombinant human MMP-2 (M9070); tris-borate EDTA buffer (TBE, T3913); and Brij 35 (B4184) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Glycerol (GX0185-6) was obtained from EMD Chemicals (Gibbstown, NJ, USA). Tris base (BP152), calcium chloride dihydrate (C79), and hydrogen chloride (A-144S) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Sodium chloride (42429) was obtained from Acros Organics (Geel, Belgium). Novex pre-cast 1-mm thick 20%T 2.6%C polyacrylamide TBE gels

were obtained from Invitrogen. Phosphate buffered saline (PBS, 2810305) was obtained from MP Biomedicals (Solon, OH, USA).

For experiments testing fresh samples, peripheral blood was collected from volunteers into vacutainers containing heparin anticoagulant (BD Biosciences, San Jose, CA, USA). Then, heparin plasma was obtained from these blood samples by collecting the supernatant after centrifugation at either 1600g for 15 minutes (diabetes trials 1-3) or at 1000g for 15 minutes (physiological shock trial). For experiments testing stored, frozen samples, the samples were either heparin plasma (diabetes trial 4) or EDTA plasma (pancreatic cancer trial). The EDTA plasma samples had undergone one prior freeze thaw cycle. Whole blood and plasma samples for diabetes trials 1-4 were obtained from Dr. Karen Herbst (Department of Medicine, VA Medical Center, UCSD). Whole blood and plasma samples for physiological shock experiments were obtained from Dr. Alexander Penn and Dr. Geert W. Schmid-Schönbein (Department of Bioengineering, UCSD). Plasma samples for pancreatic cancer testing were obtained from the UCSD Biorepository (Dr. Richard B. Schwab, Department of Medicine, UCSD).

5.2.2 Type II Diabetes Trials

A stock solution of 1 mg/mL of the substrate S4 was prepared in 1X PBS (pH 7.8). Selected dilutions of MMP-2 were prepared in TCNB buffer (50 mM Tris (pH 7.5) with 10 mM $CaCl_2$, 150 mM NaCl, and 0.05% Brij 35). In all 4 of the diabetic trials performed (trials 1-4), blood and plasma reactions were achieved by mixing 1:1 with 1 mg/mL substrate S4. Enzyme standards were obtained by spiking specific concentrations

of MMP-2 in a 0.5 mg/mL solution of 1X PBS. For all reactions (blood, plasma, or 1X PBS standards), the final substrate concentration was 0.5 mg/mL. For diabetes trials 1-3, the final enzyme standard concentrations were: 20, 6, 1, 0, and 0 nM. For diabetes trial 4, the final enzyme standard concentrations were: 6, 3, 1 and 0 nM. Reactions were allowed to proceed for 30 minutes for trials 1-3 and for 1 hour for trial 4. After completion of protease reactions, aliquots of 6 μL of these mixtures were loaded into 8x8x0.1 cm pre-cast Novex 20%T 2.6%C vertical polyacrylamide TBE gels. After electrophoresis at 500V for 10 minutes, the gels were imaged directly by a BioDoc-It System with a Model M-26 transilluminator (UVP, Upland, CA, USA) at an excitation of 302 nm and emission of 500-580 nm. Gel fluorescence was then quantified with a Storm 840 gel scanner (Molecular Dynamics, Sunnyvale, CA, USA) (fluorescence mode, high sensitivity, 100 μm pixel size, 1000V photomultiplier tube) with a 450 nm excitation filter and a 520 nm long-pass emission filter. The fluorescent signal was obtained using ImageQuant v5.2 by integrating the fluorescence over the entire cleavage product band. In Trials 1-4, the following types of samples were tested:

- Diabetes trial 1. Fasting whole blood and heparin plasma from n=5 normal healthy patients and from n=5 patients with type II diabetes.
- Diabetes trials 2-3. Blood and plasma from a healthy patient (n=1) and from a type II diabetic (n=1) at 0, 60, 120, and 180 minutes time points during an oral glucose tolerance test (OGTT).
- Diabetes trial 4. Frozen Li^+-heparin plasma samples (t=0 minutes from an OGTT) from healthy controls (n=11), from placebo-treated type II diabetics at day 1 (n=6), from

doxycycline-treated type II diabetics at day 1 (n=6), from placebo-treated type II diabetics at day 84 (n=6), and from doxycycline-treated type II diabetics at day 84 (n=6).

5.2.3 Physiological Shock Trial

Stock solutions of 1 mg/mL of the substrates S1 and S3 were prepared in 1X PBS (pH 7.8). Selected dilutions of trypsin and α-chymotrypsin were prepared in 1 mM HCl.

Dr. Alexander Penn obtained blood and plasma samples before and after he induced physiological shock in 4 healthy adult Wistar rats (rats 1-4, respectively: 364 g, 433 g, 345 g, 419 g). First, pre-shock samples were collected to establish normal levels of protease activity. Hemorrhagic shock was induced by drawing blood at 2 mL/minute until the blood pressure reached 35 mm Hg (after approximately 30 minutes). This blood pressure was then maintained for 2 hours. Next, the entire volume of extracted blood (minus that collected for sample testing) was returned to the animal at 0.5 mm Hg/minute. Since 2 mL blood had been collected earlier for further analysis, 2 mL saline was perfused into the animal in order to compensate for this lost volume. At 15 minutes after reperfusion was completed, blood and plasma samples were obtained to provide post-shock sample.

Measurements of α-chymotrypsin-like and trypsin-like activity in blood and plasma were achieved by mixing the samples 1:1 with 1 mg/mL of substrate S3 and substrate S1, respectively. Enzyme standards were obtained by spiking specific concentrations of α-chymotrypsin and trypsin in a solution of 0.5 mg/mL substrate S3 and substrate S1, respectively, in 1X PBS. The reactions proceeded for 30 minutes and

were then loaded 6 μL/well into 20% pre-cast polyacrylamide gels and electrophoresed at 500V for 10 minutes. Imaging of the gels and quantification of fluorescence were performed as described in Section 5.2.2.

5.2.4 Pancreatic Cancer Trial

Stock solutions of 1 mg/mL of the substrates S1, S2, and S3 were prepared in 1X PBS (pH 7.8). Selected dilutions of trypsin and α-chymotrypsin were prepared in 1 mM HCl. Dilutions of elastase were prepared in 100 mM Tris-HCl (pH 8.0). Frozen EDTA plasma samples were obtained from 10 pancreatic cancer patients, 10 normal healthy patients, and 1 pancreatitis patient (UCSD Biorepository). Enzyme measurements in blood and plasma were achieved by mixing the samples 1:1 with 1 mg/mL of the following substrates: (a) α-chymotrypsin with S3, (b) trypsin with S1, and (c) elastase with S2. Enzyme standards were obtained by spiking specific concentrations of enzyme in a solution of 0.5 mg/mL of their respective substrates in 1X PBS. The reactions proceeded for 1 hour and were then loaded 6 μL/well into pre-cast 20% polyacrylamide gels. For each enzyme, two gels (each 15-well capacity) were used to fit all 21 samples and 9 enzyme standards. After electrophoresis at 500V for 10 minutes, imaging and quantification were performed as described in Section 5.2.2.

5.3 Results and Discussion

5.3.1 MMP-2-like Activity in Type II Diabetics

In diabetes trial 1, the MMP-2/9 substrate, S4, was used to measure MMP-2-like activity in normal and type II diabetic patients. Figures 5.1A-B show the resulting gels for detection of MMP-2-like activity in whole blood and heparin plasma, respectively, from n=5 healthy normal controls and n=5 type II diabetic patients. Figures 5.1C shows the fluorescence data from individual samples and Figure 5.1D summarize this data by presenting the average fluorescence values for each sample type. MMP-2-like activity in blood was decreased in diabetes type II (DM2) samples, relative to control samples (CTL), though difference were not statistically significant ($p=0.43$). Plasma MMP-2-like activity was increased in DM2 samples. Though these differences were also not significant ($p=0.23$), there were greater differences in the mean fluorescence value. Since the literature has repeatedly shown that there are elevated concentrations of MMP-2 (and sometimes MMP-9) [19, 21, 23] in the plasma of patients with type II diabetes, it is likely that the small decrease observed in blood can simply be attributed to the low number of samples included in this study. This is further supported by the additional sample testing in subsequent experiments (diabetes trials 2-4), which verify that MMP-2-like activity is generally increased in type II diabetic blood. Regarding the difference between blood and plasma measurements, it is likely that the differences between the DM2 and CTL patients were larger (in magnitude) in plasma than in blood because of lower nonspecific cleavage in plasma (blood has a higher concentration of proteases).

For comparison, YSI glucose measurements (provided by Dr. Karen Herbst) are also presented in Figures 5.1C-D. Glucose levels can be classified into the following groups according to the American Diabetes Association: 70-99 mg/dL, normal fasting glucose; 100-125 mg/dL, pre-diabetes; and ≥126 mg/dL, diabetes. Several of the DM2 and CTL patients had anomalous glucose values. For example, one of the CTL patients was below normal levels at 62.3 mg/dL and another CTL patient was at pre-diabetic levels at 101 mg/dL. One of the DM2 patients was at a pre-diabetic level of 125 mg/dL and another DM2 patient was at a normal level of 94.5 mg/dL. Thus, it is possible that the trend in normal and diabetics will be more evident in future studies with more control over patient inclusion, based on fasting glucose levels.

5.3.2 MMP-2-like Activity During an Oral Glucose Tolerance Test

It is possible that diet may affect the MMP activity levels in patients with type II diabetes. Thus, these next experiments were designed to measure MMP activity for several time points after consumption of glucose (75 g), during a clinical test known as an oral glucose tolerance test (OGTT). The goal of an OGTT is to determine a person's insulin resistance by his or her ability to remove glucose from the blood. However, the current trial will instead use OGTT samples to track changes in MMP activity over time. In diabetes trials 2-3, MMP-2-like activity was measured in 1 diabetic and in 1 normal control at t=0, t=60, t=120, and t=180 minutes after administration of glucose. Figures 5.2A and 5.3A show the gel results for detection in whole blood for diabetes trials 2 and 3, respectively. Figures 5.2B and 5.3B show the gels for detection in heparin plasma in

those respective trials. Figure 5.2C summarize the fluorescent signals measured in the gels of Figures 5.2A-B and Figure 5.3C summarizes the fluorescent signals measured in the gels of Figures 5.3A-B. In diabetes trial 2, the DM2 patient signal was higher than the CTL patient signal, in both blood and plasma (Figure 5.2C). When comparing the time course observed in the diabetic to that seen in the normal control, both exhibited similar trends, whether in plasma or blood. There was an increasing signal with time for both DM2 and CTL blood samples and a decreasing signal with time for both the CTL and DM2 plasma samples. The greatest difference in signal between the CTL and DM2 groups was at t=180 minutes for blood and t=0 minutes for plasma. In diabetes trial 3, the DM2 signal was higher than the CTL signal in both blood and plasma (Figure 5.3C). There was no obvious trend for the time course for either the CTL or DM2 patient, in either blood or plasma. In plasma, there was an almost level trend (slightly increasing in the CTL patient, slightly decreasing in DM2 patient). In blood, there was an overall increasing trend in both the CTL and DM2 patients after a linear fitting (not shown). The greatest difference in signal between the CTL and DM2 patient was observed at t=120 minutes for blood and t=0 minutes for plasma. Thus, both studies indicate that a later time point (120-180 minutes) is best for differentiating diabetic and healthy blood samples and that an earlier time point (t=0 minutes) is best for differentiating the two in plasma. Both studies also clearly show elevation of MMP-2-like activity in the diabetic for all time points, which is consistent with prior studies [19, 21, 23].

In a similar study to that above by Derosa et al., MMP-2 and -9 levels were measured in EDTA plasma by ELISA during an OGTT [21]. The most significant difference from the levels observed at t=0 minutes (for both enzymes) was observed at

the longest time point tested, t=180 minutes. The biggest difference between the diabetic and the normal controls was also observed at this same time point. Thus, this evidence would suggest that the t=180 minutes time point is the best choice for future experiments (that use OGTT samples). Based on the findings of diabetes trials 2-3, however, the longer time points were only best for blood and not for plasma in terms of resolving diabetic from normal samples. With further sample testing with the charge-changing substrate assay, it will likely become more evident the longer time points are best. However, given the *small differences* observed amongst the different time points, we concluded that the t=0 minute samples would be sufficient for diabetes trial 4.

Again for comparison, YSI glucose measurements (provided by Dr. Karen Herbst) are presented in Figures 5.2C and 5.3C. The CTL and DM2 fasting glucose values (t=0 minutes) were 96.6 mg/dL and 223 mg/dL, respectively, for diabetes trial 2, and were 78.8 mg/dL and 132 mg/dL, respectively, for diabetes trial 3. The fasting glucose values for both the CTL patients and DM2 patients fell within the normal range (70-99 mg/dL) and diabetic range (>126 mg/dL), respectively (see 5.3.1 for fasting level classification). In addition to fasting glucose levels, patients can also be classified, according to the American Diabetes Association, by the levels of glucose at t=120 minutes of an OGTT: <140 mg/dL, normal glucose tolerance; 140-200 mg/dL, pre-diabetes; and ≥200 mg/dL, diabetes. The CTL and DM2 t=120 minutes glucose values were 65.2 mg/dL and 375 mg/dL, respectively, for diabetes trial 2, and were 84.9 mg/dL and 218 mg/dL, respectively, for diabetes trial 3. The t=120 minutes levels for both the CTL and DM2 patients thus fell within the expected normal range (<140 mg/dL) and the diabetic range (≥200 mg/dL), respectively. However, it should be noted that the glucose

values were, on average, much higher in diabetes trial 2 than in diabetes trial 3 for both the CTL and DM2 patients. In addition, the MMP activity was also, on average, higher for both patients of diabetes trial 2. This higher glucose level may be indicative of a higher insulin resistance in both of the patients of diabetes trial 2 caused by an elevated level of MMP activity. Insulin resistance would subsequently lead to higher glucose levels in the blood. With greater insulin resistance in the CTL patient of diabetes trial 2 (versus in diabetes trial 3), this provides a possible explanation for why there was a much greater difference between the CTL and DM2 patients in diabetes trial 3.

5.3.3 MMP-2-like Activity in Diabetics Treated with Doxycycline

In diabetes trial 4, substrate S4 was used to measure MMP-2-like activity in type II diabetic patients that had either been treated with an MMP inhibitor, doxycycline (doxy), or that were treated with a placebo. These activities were measured in samples that were collected at the beginning of the study (day 1, baseline) and at the end of a 12-week course of treatment (day 84, end of study), with normal healthy patients included as an additional control. The normal patients never received any treatment. In total, this study included 5 different groups: 11 normal controls (control group), 6 placebo-treated diabetics at day 1 (p1 group), 6 doxy-treated diabetics at day 1 (d1 group), 6 placebo-treated diabetics at day 84 (p84 group), and 6 doxy-treated diabetics at day 84 (d84 group). Since the pre-cast gels used in this experiment can only handle 15 samples per gel, all 35 samples were split amongst 3 separate 20% polyacrylamide gels, gels 1-3. The remaining wells were loaded with 1X PBS enzyme standards (different concentrations of

enzyme reacted in buffer). Figures 5.4A-C show the resulting gel for the comparison of all 5 groups in gels 1-3. In gel 4, shown in Figure 5.4D, 6 samples from the control group were compared to the 6 samples from the p1 group. Figure 5.4E shows the fluorescence values for all the individual samples loaded into gels 1-3. Figure 5.4F summarizes the data of Figure 5.4E by presenting the average fluorescence values for all 5 groups, combining the data from gels 1-3. Figure 5.4G summarizes the average fluorescence values of the 5 groups in gels 1-4, individually, without combining the data from the separate gels (showing gel-to-gel variation). The results of this experiment show that diabetic patient plasma samples had elevated MMP-2-like activity versus normal controls. In gels 1-3, there was a statistically significant ($p < 0.05$) difference between the control and the p1, p84, and d84 diabetic groups (see Figure 5.4F). In gel 4, there was a significant difference between the control group and the p1 diabetic group (see Figure 5.4G). Furthermore, the experiment showed that placebo-treated samples had higher activity than doxy-treated samples, with greater differences at day 84. This indicates that the doxcycline treatment is having the intended effect of blocking MMP activity. Finally, there was a significant difference between the d1 groups in gel 1 versus in gel 3, which may be due to patient-to-patient variability (see Figure 5.4G).

In another finding of the experiment, day 84 (p84, d84) sample values had higher activity than day 1 (p1, d1) values, which in turn had higher activity than the controls. Sample age may be a factor in these results. The 5 groups have different sample ages and were stored in a freezer for different lengths of time (see Figure 5.4H). On average, the control samples were ~1 month older than the day 1 samples, which were in turn ~2-3 months older than the day 84 samples. Samples that were stored in a freezer for a shorter

period of time will experience less degradation and have more enzyme activity. Thus, day 84 samples, stored for less time, should have more activity than the day 1 samples, even with doxcycline treatment. Thus, this lends support to the notion that it best to avoid sample preparation and subsequent sample storage. Protease activity is measured more accurately by testing fresh samples immediately, or as soon as possible, after collection.

YSI glucose measurements, provided by Dr. Karen Herbst, support the hypothesis that doxycycline is a potential therapeutic for type II diabetes. Figure 5.4E presents the glucose levels for individual samples and Figure 5.4F presents the average levels for each group. Recalling the normal, pre-diabetic, and diabetic ranges provided in Chapter 5.3.1, the control group (n=11) had 9 normal patients and 2 pre-diabetic patients. For the p1 and p84 group (both n=6), all the patients were classified as diabetic. For the d1 group (n=6), 4 patients were diabetic and 2 patients were pre-diabetic. For the d84 group (n=6), 3 patients were diabetic, 2 patients were pre-diabetic, and 1 patient was normal. As was the case in Chapter 5.3.1, some of the members of the groups fell outside the desired ranges for this study, with small portions of the control group and the day 1 diabetic groups (p1, d1 groups) having pre-diabetic members. The differences between each group should become more evident with a greater control over patient inclusion in future studies (e.g. having only normal patients included into the control group). On average for each group, the glucose values were 91 mg/dL, 175 mg/dL, 142 mg/dL, 184 mg/dL, and 132 mg/dL for the control, p1, d1, p84, and d84 groups, respectively. This further confirms the observed effect of doxycycline treatment, showing an increase in average glucose values for placebo-treated patients (p1 vs. p84) and a decrease in average glucose values for doxycycline-treated patients (d1 vs. d84) over the course of the study. It is possible that

with a longer course of treatment, or with a higher dosage, that this improvement would be even greater. Summarizing, the data presented in Figure 5.4 supports the hypothesis that doxycycline reduces MMP activity and subsequent development of insulin resistance, showing a decrease in both blood glucose values and MMP activity in doxycycline-treated diabetics, compared to placebo-treated diabetics, after the end of the 84-day course of treatment. Further testing may confirm that doxycycline is a potential course of treatment for type II diabetes.

5.3.4 Physiological Shock Trial

In the physiological shock trial, charge-changing substrates were used to measure differences in protease activity between pre-shock and post-shock blood and plasma samples obtained from a hemorrhagic shock model in adult Wistar rats (n=4). Specifically, substrates S3 and S1 were used to measure differences in chymotrypsin-like and trypsin-like activity, respectively. Figures 5.5A-B show representative gel patterns for the respective detection of activity for these two enzymes, in a single rat. Figure 5.5C shows the increase in activity in the blood and plasma of all 4 of the rats studied, providing a ratio (post/pre) of the post-shock and pre-shock protease activities. As an example, a post/pre ratio of 1 means the activity did not increase and a post/pre ratio greater than 1 indicates that protease activity increased. The average post/pre ratios are presented in Figure 5.5D. The results of the charge-changing substrate assay showed that the trypsin-like activity increased 10% in blood and increased 25% in plasma, relative to pre-shock values. Chymotrypsin-like activity increased 4% in blood and increased 63%

in plasma. A paired t-test between pre-shock and post-shock values did not indicate a significant difference ($p< 0.05$) for n=4, although the number of rats tested were small. Given the limited resources for this study, this small proof-of-concept study (which is not a clinical trial) gives a preview of the possibility that this assay can be used for shock diagnosis. Clearly, this assay is picking up elevated chymotrypsin-like and trypsin-like activity during physiological shock. This is particularly encouraging considering this assay achieved such detection in 6 μL of unprocessed whole blood in approximately 40 minutes.

Chromogenic substrate measurements provide further support that there is elevated pancreatic protease activity in the blood during physiological shock. In a study performed by Dr. Alexander Penn, the chromogenic substrates BAPNA (N_{α}-Benzoyl-L-arginine 4-nitroanilide hydrochloride), SPNA (N-Succinyl-L-phenylalanine-p-nitroanilide), and SAAPLPNA (N-Suc-Ala-Ala-Pro-Leu-pNA) were used to measure trypsin-like, chymotrypsin-like, and elastase-like activity, respectively, in heparin plasma. Figure 5.6 shows the absorbance measurements for this assay at t=90 minutes, t=180 minutes, and t=1440 minutes (24 hours). Early time points (t=90, t=180 minutes) showed that trypsin-like and chymotrypsin-like activity decreased post-shock. At 24 hours, however, both activities were elevated by approximately 51% and 27%, respectively. It should be noted that none of these elevations were statistically significant. Elastase-like (SAAPLPNA) activity significantly ($p<0.05$) increased post-shock at all time points, with a very large increase of 370% at 24 hours. There are several important points to make based on the results of these chromogenic assays. First, the 24-hour time points confirm the elevation of chymotrypsin-like and trypsin-like activity that was

observed with the charge-changing substrates. Second, the elastase-like activity is significantly elevated at all time points, showing bigger differences between post- and pre-shock samples than for chymotrypsin-like and trypsin-like activity. It is apparent that elastase may be a better marker than the other two enzymes. Thus, future experiments could benefit from using a charge-changing elastase substrate (e.g. substrate S2) to differentiate pre- and post-shock samples. Third, with the exception of elastase, it takes 24 hours for the chromogenic substrate assay to detect elevated protease activity. The charge-changing substrate assay only needed approximately 40 minutes. The long assay times for the chromogenic substrates make them unsuitable for a shock diagnostic. However, charge-changing substrates achieve rapid detection and are well suited for this important goal.

The above data, presented in Figures 5.5-5.6, does not take into account blood dilution. For example, the animal shifts fluid into the vascular space to compensate for lost blood volume during the ischemic period. This could manifest as a higher blood pressure after reperfusion, as observed by Dr. Alexander Penn. His blood pressure measurements show a statistically significant elevation 30 minutes post-shock (see Figure 5.6 inset). However, there are various reasons that blood pressure can increase without dilution (e.g. vasoconstriction), so it would be difficult to account for blood dilution due to this phenomenon.

Another type of blood dilution, which can readily be taken into account, occurs due to a saline perfusion step (see Chapter 5.2.3). Approximately 2 mL saline is returned to the rat during reperfusion to replace the blood withdrawn for testing. Since rat blood is approximately 50% blood cells and 50% plasma, the return of just saline (without blood

cells) leads to a dilution of the plasma, affecting the measured level of protease activity in post-shock samples. Appendix 2 derives a dilution factor that can be used to correct the fluorescent signal for post-shock samples. Figure 5.7 shows the data of Figure 5.5 with this important correction, with post-shock values increasing an average of 1.1-fold. The corrected data for the charge-changing substrate assay shows that trypsin-like activity was increased 19% in blood and increased 36% in plasma, relative to pre-shock samples. Chymotrypsin-like activity increased 13% in blood and increased 78% in plasma. A paired t-test between pre- and post-shock values still did not indicate a significant difference ($p < 0.05$) for either enzyme in blood or plasma. Figures 5.8 shows the data of Figure 5.6 after correction for saline-based dilution. The corrected data for the chromogenic substrate assay shows an even larger difference between pre-shock and post-shock chymotrypsin-like and trypsin-like activity. Unfortunately, this was not enough to reach statistically significant differences (for $n=4$). Elastase-like activity, already having a statistically significant increase before correction, did not gain additional significance ($p=0.02$, before and after correction). It is likely that additional sample testing will achieve statistical significance in both assays for the elevations of all three enzymes in both blood and plasma. Using a power calculation, it is estimated that an additional 9-13 rats (depending on the enzyme) are needed to achieve statistical significance (for data with the dilution correction).

5.3.5 Pancreatic Cancer Trial

In the final study, charge-changing substrates were used to measure pancreatic protease activity in EDTA-treated frozen plasma samples from 10 pancreatic cancer patients, 10 healthy patients, and 1 pancreatitis patient. Specifically, substrates S1, S2, and S3 were used to measure differences in trypsin-like, elastase-like, and chymotrypsin-like activity, respectively. In addition to treatment with a protease inhibitor (EDTA), these samples had previously undergone one freeze-thaw cycle and are estimated to be approximately 2 years old, on average. Considering that these conditions are *very* far from ideal, it was not expected that there would be much, if any, protease activity detected in these samples. Sample reactions were split between two gels (gel 1 and gel 2) because of the limitation that only 15 samples could be loaded per gel. Figures 5.9A-C shows the results for detection of chymotrypsin-like (substrate S3), trypsin-like (substrate S1), and elastase-like activity (substrate S2), respectively. By eye, the results show that there were not many visible differences between the normal and pancreatic cancer samples. There were a few exceptions where some of the healthy samples had elevated protease activity. For example, in gel 2 of Figure 5.9B, one of the healthy patient samples clearly had elevated trypsin-like activity, relative to the activities observed in the pancreatic cancer samples. Data analysis revealed that there was a corresponding 8%, 5%, and 9% decrease in activity in pancreatic cancer samples for these three enzymes, relative to healthy samples. A two-tailed, unpaired t-test did not show significant differences (comparing results just from individual substrates). It is likely that better results will be obtained through additional testing using fresh samples that have not been

treated with any chelators. Such samples should have greater enzyme activity and provide a much more accurate result. A future trial is currently planned with Evangeline Mose and Dr. Andrew Lowy at the UCSD Moores Cancer Center. This trial will provide whole blood samples from their genetic mouse model for pancreatic cancer and potentially open the door to the discovery of early-stage markers for this deadly disease.

5.3.6 Comparing Measurements in Blood and Plasma

In the experiments of Figures 5.1, 5.2, 5.3 and 5.5, there was a 2- to 9-fold higher signal (positively-charged fluorescent band) generated for protease detection in whole blood than in plasma. There are three potential explanations for this phenomenon:

- First, there may be a higher autofluorescent background generated from positively charged blood components. However, the evidence from Figures 3.9 and 4.5 does not support this explanation. In those experiments, there was no discernable fluorescence migrating toward the cathode in lanes that were loaded with pure whole blood (not mixed with any substrate). This makes sense since most blood components are negatively charged and migrate toward the anode. In fact, the difference between whole blood and plasma is that the former contains blood cells, which are highly negatively charged because of their lipid bilayer. Thus, charge-changing substrates, rather than blood components, are the likely source for the additional fluorescent signal in blood.
- The second possible explanation is that there is a positively charged complex that forms after non-specific binding of the (uncleaved) charge-changing substrate to blood components (e.g. substrates binding to cells). Again, this is not a likely explanation since

most blood components are negatively charged. Non-specific binding would mostly contribute to fluorescent background that migrates toward the anode. To test this hypothesis, a future experiment could characterize the amount of non-specific binding by tracking the electrophoretic migration, in whole blood, of a cleavage-resistant or non-amino acid analogue to a charge-changing substrate (e.g. using D-amino acids instead of L-amino acids). If any fluorescent band migrates toward the cathode, even after ensuring minimal substrate cleavage, this would support the possibility that there is non-specific binding.

- Finally, the most plausible explanation for the increased activity in whole blood is that there is a higher amount of protease activity in blood. Blood cells, such as erythrocytes and leukocytes, produce various proteases [150-153]. Some of these proteases are present on the outer cell-membrane and are not completely blocked by serum protease inhibitors [153]. Even if these enzymes are contained within blood cells (e.g. in the cytosol, on the inner cell-membrane surface, or in granules), some can be released through secretion (e.g. degranulation) or cell lysis. This could occur naturally (e.g. sample degradation) or due to any handling of the sample (e.g. pipetting). Since charge-changing substrates are not perfectly specific, they likely detect this higher activity from the additional proteases that are present in whole blood. In plasma, however, these enzymes are discarded when the blood cells are removed to obtain plasma or serum. Thus, it is expected that plasma activity would be generally lower.

As a consequence of the higher activity in blood, the difference in activity in diseased and healthy samples was greater in plasma than in blood for physiological shock (see Figure 5.5). Additional, non-disease related activity (e.g. from cell lysis or by

coagulation-induced degranulation) might have reduced the difference in activity between healthy and diseased samples by raising the background. To minimize this increase in background, there are several suggested precautions that should be taken in future experiments: 1) developing more specific substrates that better target *disease-related* protease activity, 2) performing protease activity measurements soon after sample collection to avoid natural sample degradation processes, and 3) optimizing sample-handling steps, such as pipetting, to minimize coagulation and cell lysis.

5.3.7 Assay Precision

In order to provide a measure of the variability encountered in the charge-changing substrate assay, Tables 5.1-5.2 present coefficients-of-variation (CVs) that were calculated using the data from diabetes trial 4 (Chapter 5.3.3) and from the physiological shock trial (Chapter 5.3.4), respectively. The CV is calculated by normalizing the standard deviation by the mean. It gives a measure of variability with respect to the mean. Lower values indicate that the assay has a greater precision, with 10%-15% being a typically good CV value.

The between-gel CV measures the variability from loading one sample in multiple gels. In diabetes trial 4, the average between-gel CV for plasma samples was approximately 39% (Table 5.1). The average between-gel CV for enzyme standards was approximately 22%. In the physiological shock trial, the average between-gel CV for enzyme standards was approximately 39% (Table 5.2). One of the limitations in using a CV to measure precision is that it is sensitive to small changes of a small signal (e.g.

from a 0 nM standard). Excluding the CV data for the 0 nM standard, the average CV then becomes approximately 23%. A within-gel CV measures the variability from loading one sample multiple times within the same gel. In the physiological shock trial, the average CV for blood measurements was 10% (Table 5.2). For plasma, the average CV was 11%. Excluding the 0 nM standard, the average within-gel CV for enzyme standards was approximately 7%. The between-gel and within-gel variability is probably mostly due to error introduced by small-volume pipetting (e.g. 6 μL for gel loading, 1 μL for enzyme spiking). If water were being pipetted, such a small volume would introduce an error of approximately 1-2%. However, blood and plasma samples are more viscous and difficult to pipette. Thus, the error may be considerably larger for the transfer of biological fluids with a pipette. Other potential sources of error include variability in gel thickness, gel density, and reaction time. For an assay in its early stage of development and using off-the-shelf components, the between-gel and within-gel CVs show that the current assay has a reasonable level of precision. The precision can be improved through the miniaturization and automation of the charge-changing substrate assay, and by integration into a point-of-care system.

5.4 Concluding Remarks

Charge-changing substrates were used to measure protease activity in whole blood and plasma from human patients with type II diabetes, from rats undergoing physiological shock, and from human patients that have pancreatic cancer. In these experiments it is repeatedly shown that these sensitive substrates are able to differential

normal from unhealthy samples, using minimal amounts of time (30 min. to 1 hour) and sample (6 μL).

In type II diabetes, statistically significant ($p<0.05$) elevations of MMP-like activity were observed relative to normal healthy patient controls. Patients treated with the MMP inhibitor doxycycline had reduced MMP-like activity versus placebo-treated patients, although these differences were not statistically significant. Further sample testing may verify that doxcycline can be used to reduce MMP cleavage of the insulin receptor and ameliorate the onset of insulin resistance and type II diabetes.

Elevated chymotrypsin-like and trypsin-like activity was observed after induction of hemorrhagic shock, with greater increases observed in plasma than in blood and greater increases observed for trypsin-like activity. Chromogenic substrate measurements in plasma (by Dr. Alexander Penn) corroborate this increase in activity for both of these enzymes. In addition, the chromogenic assay showed there was an elevation of elastase-like activity. Paired t-tests showed that some of these elevations were either close to or had reached statistical significance (chromogenic substrate measurement of elastase). Additional sample testing (~9-13 rats) should verify that pancreatic protease activity is significantly elevated in physiological shock. Future testing for all three enzymes, chymotrypsin, trypsin, and elastase, may show that these markers (individually or in combination) are useful for the early diagnosis of physiological shock.

After testing a frozen set of EDTA-plasma samples from pancreatic cancer patients, small differences were observed for chymotrypsin-like, trypsin-like, and elastase-like activity. Relative to healthy patients, there was a corresponding 8%, 5%, and 9% decrease in activity observed, which are small changes that are not statistically

significant. However, these samples were far from ideal, having been frozen for years, having undergone a freeze-thaw cycle, and having been treated with EDTA. Future testing with fresh whole blood samples may show larger differences in protease activity. This clearly demonstrates the need for testing fresh samples, and the unfortunate consequence of storing samples in a freezer.

In summary, further validation of these applications can potentially lead to several exciting opportunities: (a) a diagnostic test that can lead to making a treatment decision (MMP inhibitor) for type II diabetes, (b) the first ever rapid POC early-stage shock diagnostic, and (c) the first ever early diagnostic for deadly pancreatic cancer.

5.5 Acknowledgements

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I am sure that we are all excited at the possibility of one day seeing the first ever early shock diagnostic develop from these early trials. I cannot thank you both enough for this fantastic opportunity, which we could not have done without your support!

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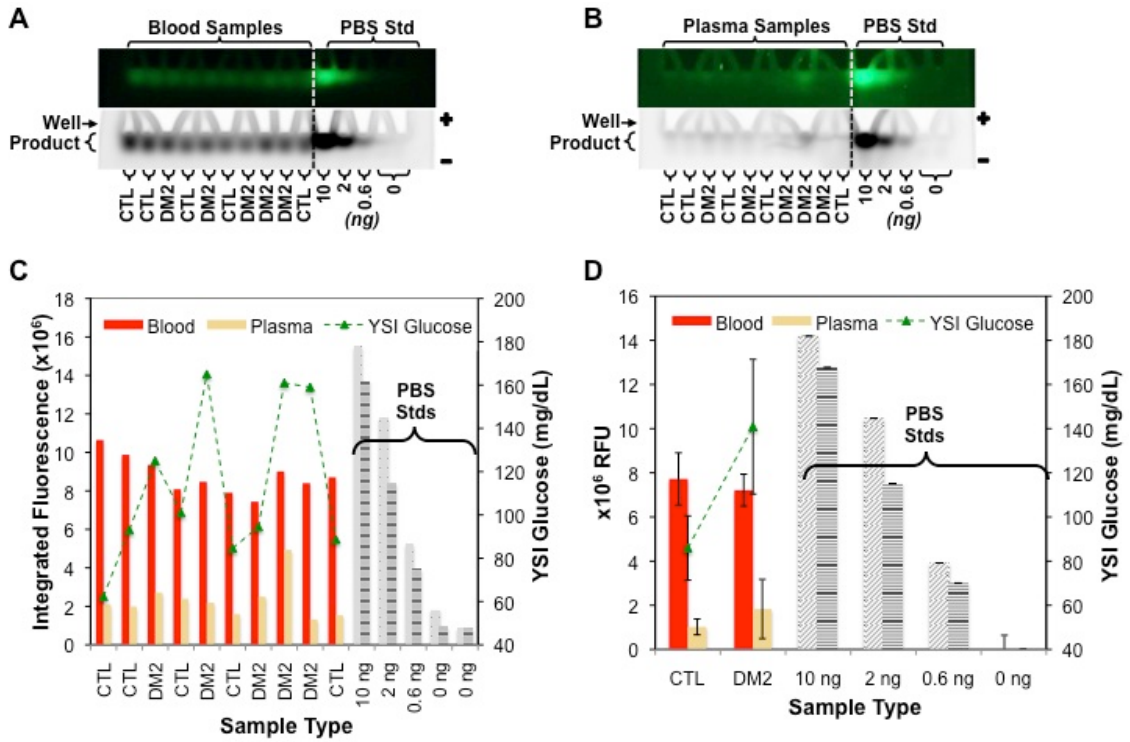


Figure 5.1: Detection of MMP-2-like activity in type II diabetes using a charge-changing substrate (diabetes trial 1). (A)-(B) Shows photos (upper image) and quantitative gel scans (lower image) for the electrophoresis patterns generated from detection of MMP-like activity in (A) whole blood and (B) heparin plasma samples obtained from diabetic patients (DM2) and normal healthy control patients (CTL). For lanes to the left of the dashed line, blood and plasma samples are mixed 1:1 with 1 mg/mL of substrate S4 (final concentration 0.5 mg/mL). The lanes to the right of the dashed line are standards in buffer obtained by spiking various concentrations of MMP-2 into a solution of 0.5 mg/mL substrate in 1X PBS. All reactions are allowed to proceed for 30 minutes and are then loaded into 1-mm thick 20% polyacrylamide gels at 6 µL/well. (C) Integrated fluorescence values corresponding to gels of (A)-(B) for blood samples (red), plasma samples (tan), and for the sets of PBS standards from both gels (grey diagonal lines, grey horizontal lines). In addition, glucose concentrations are also plotted for each patient (green triangles, provided by Dr. Karen Herbst) (D) Summary of average fluorescence values for the graph in (C), expressed in millions of relative fluorescence units (RFU).

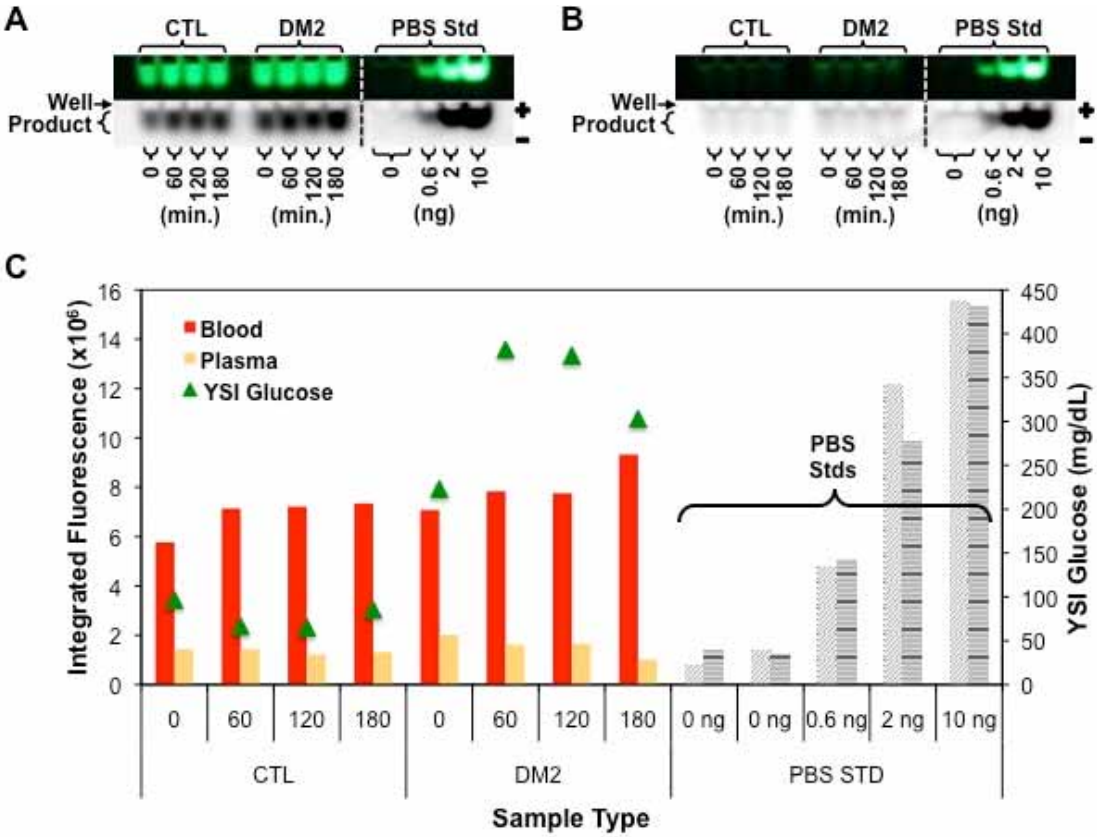


Figure 5.2: MMP-2-like activity during an oral glucose tolerance test (OGTT) (diabetes trial 2). (A)-(B) Shows photos (upper image) and quantitative gel scans (lower image) for the electrophoresis patterns generated from the detection of MMP-like activity in (A) whole blood and (B) heparin plasma samples obtained from a diabetic (DM2) and a normal healthy control (CTL) at different time points during an oral glucose tolerance test (OGTT). For lanes to the left of the dashed line in (A)-(B), blood and plasma samples are mixed 1:1 with 1 mg/mL of substrate S4. The lanes to the right of the dashed line are standards in buffer obtained by spiking various concentrations of MMP-2 into a solution of 0.5 mg/mL substrate in 1X PBS. All reactions are allowed to proceed for 30 minutes and are then loaded into 1-mm thick 20% polyacrylamide gels at 6 μL/well. (C) Integrated fluorescence values corresponding to gels of (A)-(B) for blood samples (red), plasma samples (tan), and for the sets of PBS standards from both gels (grey diagonal lines, grey horizontal lines). In addition, glucose concentrations are also plotted for each patient (green triangles, provided by Dr. Karen Herbst).

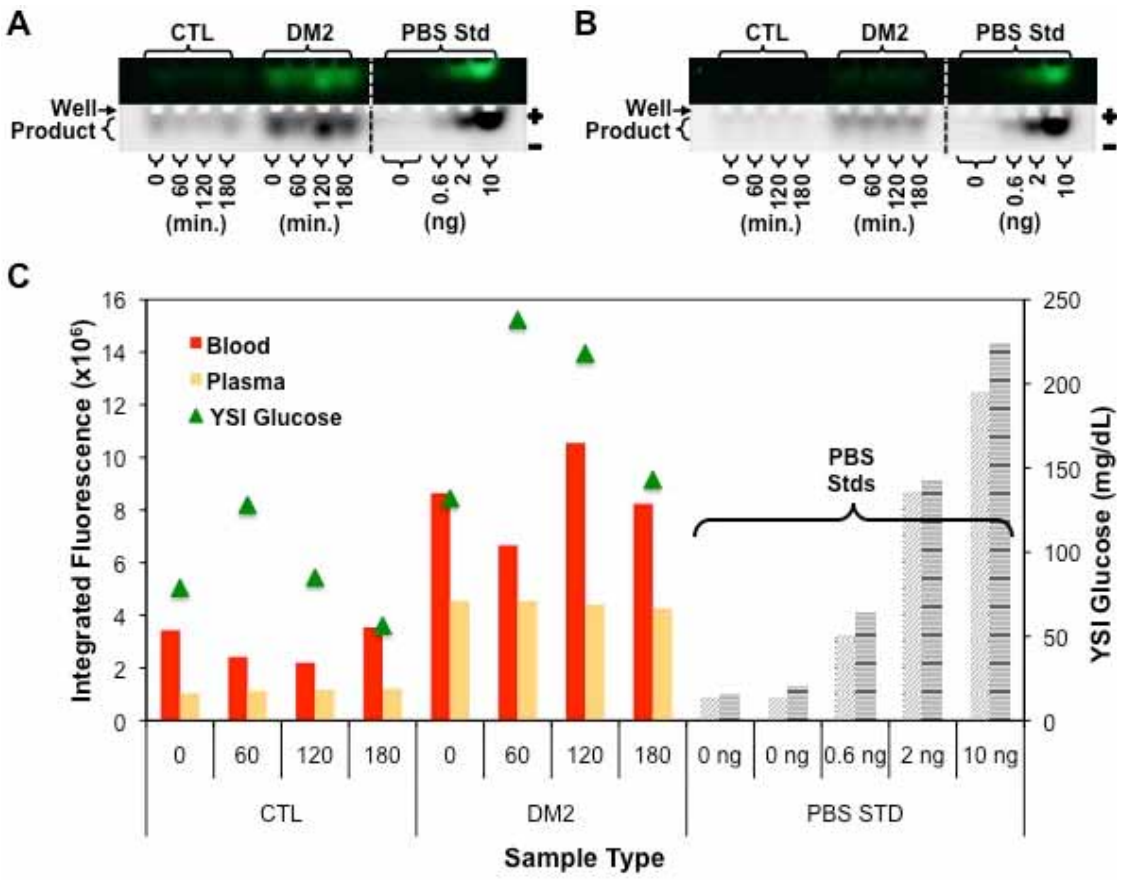


Figure 5.3: MMP-2-like activity during an oral glucose tolerance test (OGTT) (diabetes trial 3). (A)-(B) Shows photos (upper image) and quantitative gel scans (lower image) for the electrophoresis patterns generated from detection of MMP-like activity in (A) whole blood and (B) heparin plasma samples obtained from a diabetic (DM2) and a normal healthy control (CTL) at different time points during an oral glucose tolerance test (OGTT). For lanes to the left of the dashed line in (A)-(B), blood and plasma samples are mixed 1:1 with 1 mg/mL of substrate S4. The lanes to the right of the dashed line are standards in buffer obtained by spiking various concentrations of MMP-2 into a solution of 0.5 mg/mL substrate in 1X PBS. All reactions are allowed to proceed for 30 min. and are then loaded into 1-mm thick 20% polyacrylamide gels at 6 μL/well. (C) Integrated fluorescence values corresponding to gels of (A)-(B) for blood samples (red), plasma samples (tan), and for the sets of PBS standards from both gels (grey diagonal lines, grey horizontal lines). In addition, glucose concentrations are also plotted for each patient (green triangles, provided by Dr. Karen Herbst).

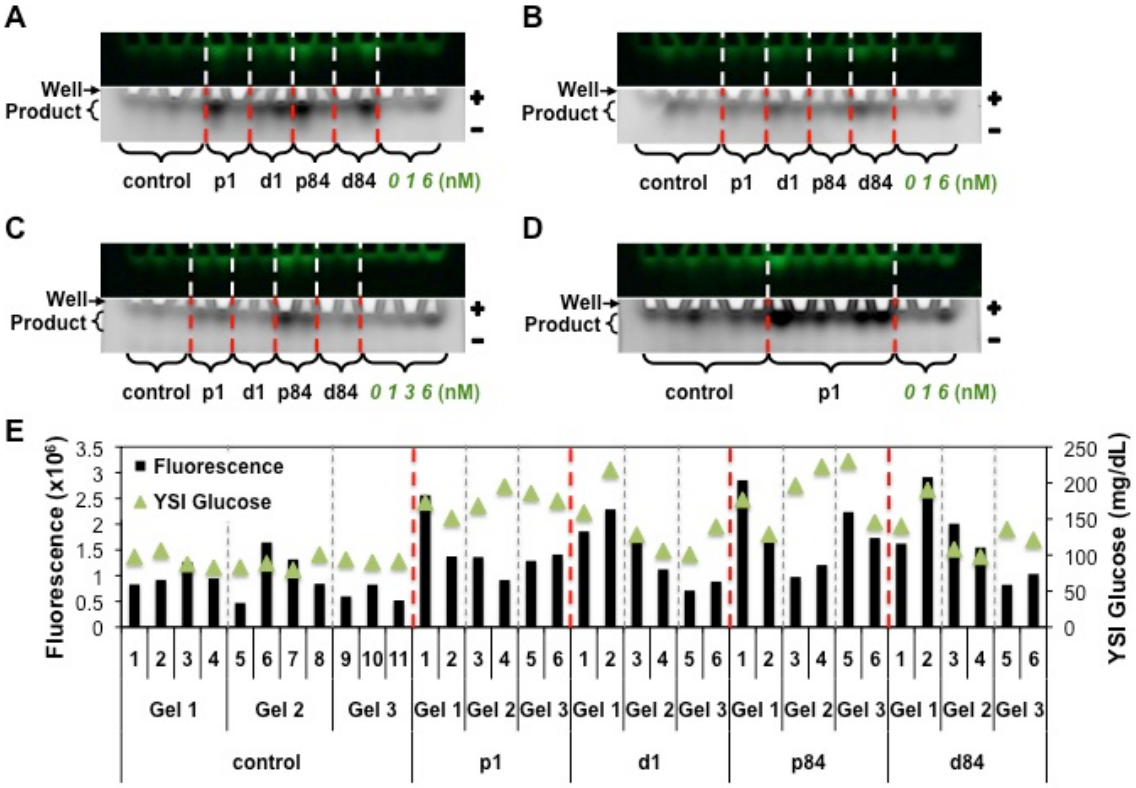
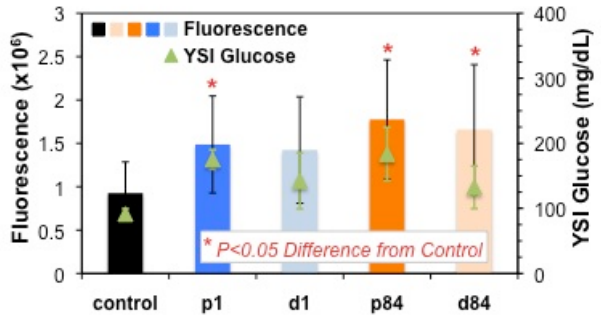
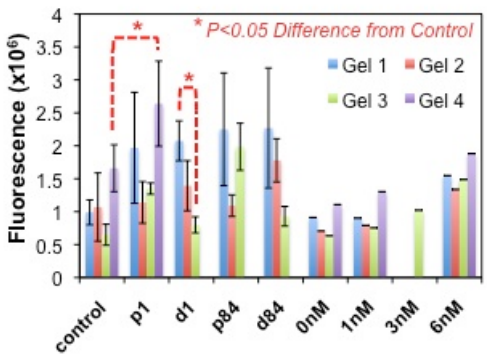


Figure 5.4A-E: MMP-2-like activity in diabetics treated with doxycycline (diabetes trial 4). (A)-(C) Gels 1-3, respectively, are electrophoresis patterns generated from the detection of MMP-like activity in heparin plasma from 5 different population groups that were split amongst three gels: n=11 normal healthy controls (control), n=6 placebo-treated patients at day 1 (p1), n=6 doxycycline-treated patients at day 1 (d1), n=6 placebo-treated patients at day 84 (p84), and n=6 doxycycline-treated patients at day 84 (d84). 1X PBS standards were included in the rightmost 3 lanes of each gel. (D) Gel 4 is a comparison of 6 control and 6 p1 patients in a single gel. In each case (A)-(D), detection was achieved after 1 hour reactions with final concentrations of 0.5 mg/mL substrate S4. This was followed by electrophoresis in 20% polyacrylamide gels (6 μL/well loading). (E) Shows fluorescence values (black) for all individual patient samples tested amongst the gels of (A)-(C). In addition, glucose concentrations are also plotted for each patient (green triangles, provided by Dr. Karen Herbst).

F



G



H

Group	Age (days) (mean ± s.d.)
control	161 ± 88
p1	127 ± 37
d1	135 ± 39
p84	42 ± 34
d84	59 ± 52

Figure 5.4F-H: MMP-2-like activity in diabetics treated with doxycycline (diabetes trial 4) (cont.). (F) Shows the average fluorescence values obtained for the 5 test groups, summarizing the combined data from gels 1-3. In addition, glucose concentrations are also plotted for each patient (green triangles, provided by Dr. Karen Herbst). (G) Shows the average fluorescence values obtained for the 5 test groups in gels 1-4, individually. (H) Shows the distribution of sample age (in days) for the 5 different test groups, providing an indication for the different lengths of time that samples were stored in a freezer.

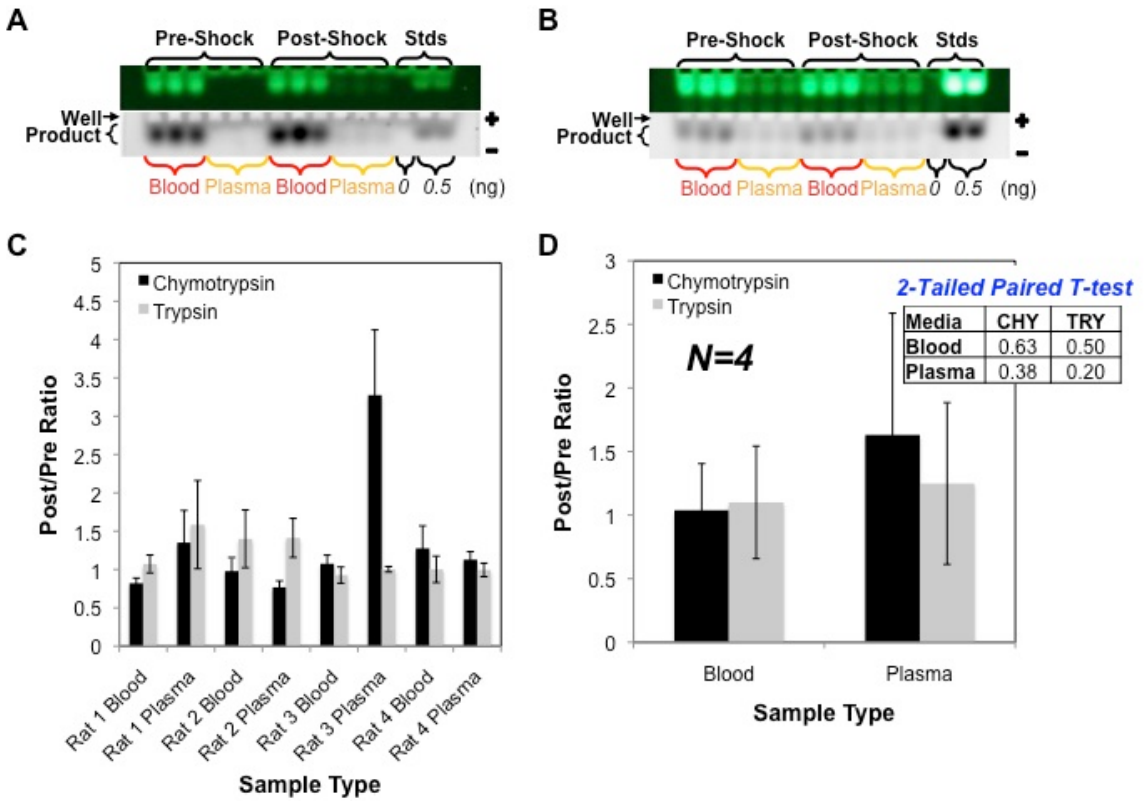


Figure 5.5: Chymotrypsin- and trypsin-like activity in physiological shock. (A)-(B) Shows photos (upper image) and quantitative gel scans (lower image) for representative electrophoresis patterns generated from the detection of (A) chymotrypsin-like and (B) trypsin-like activity in whole blood and heparin plasma from a single rat before (pre-shock) and after induction of physiological shock (post-shock). Chymotrypsin and trypsin measurements were achieved by 30-minute reactions with 0.5 mg/mL of substrate S3 and substrate S1, respectively. 1X PBS standards are included in rightmost 3 lanes in each gel. All reactions were then electrophoresed in 20% polyacrylamide gels (6 µL/well loading) (C) Shows the ratio of post-shock to pre-shock fluorescence values observed for the measurement of both enzymes in the blood and plasma of 4 individual rats. (D) Summarizes the mean values for results in (C), showing the p-values for a 2-tailed paired t-test between the pre- and post-shock values.

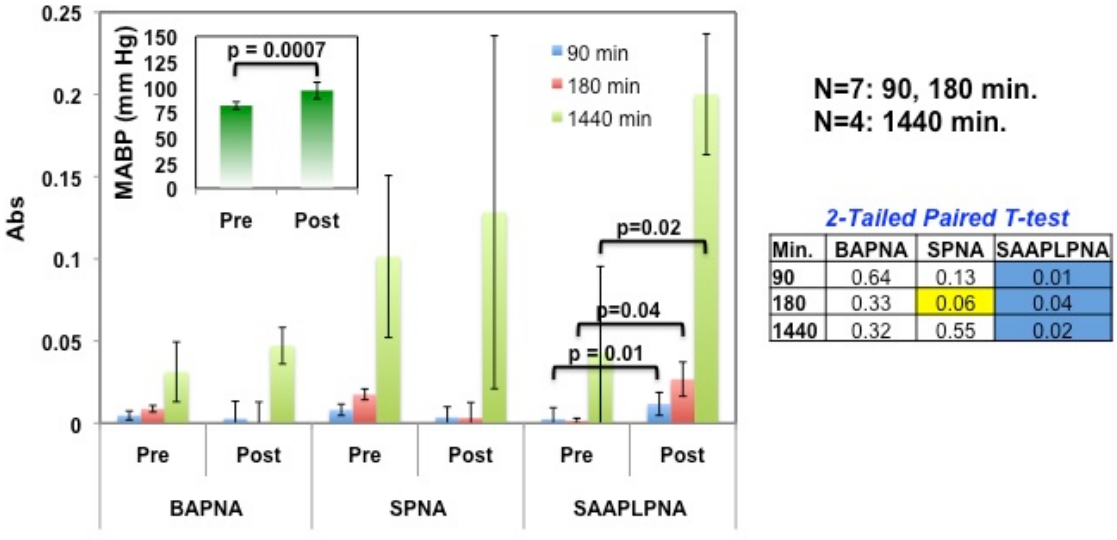


Figure 5.6: Chromogenic substrate measurements in physiological shock (measurements by Dr. Alexander Penn). Shows measured absorbance values of the t=90 minutes, t=180 minutes, and t=1440 minutes time points from chromogenic assays using BAPNA (measuring trypsin-like activity), SPNA (measuring chymotrypsin-like activity), and SAAPLPNA (measuring elastase-like activity) for both pre- and post-shock heparin plasma samples. The table on the right summarizes 2-tailed paired t-tests comparing the pre- and post-shock chromogenic substrate measurements. The inset graph shows the mean arterial blood pressure (MABP) for both pre- and post-shock samples.

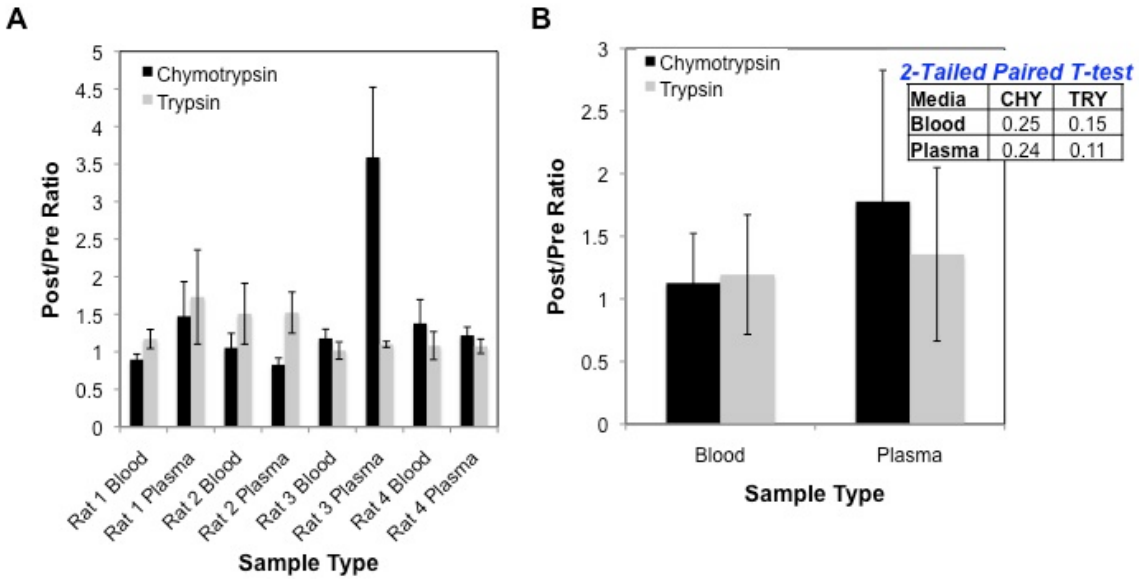


Figure 5.7: Corrected chymotrypsin- and trypsin-like activity in physiological shock. This is the same data as shown in Figure 5.5, but with a correction for post-shock sample dilution with saline. (A)-(B) Shows photos (upper image) and quantitative gel scans (lower image) for representative electrophoresis patterns generated from the detection of (A) chymotrypsin-like and (B) trypsin-like activity in whole blood and heparin plasma from rats before (pre-shock) and after induction of physiological shock (post-shock). Chymotrypsin and trypsin measurements were achieved by 30 minutes reactions with 0.5 mg/mL of substrate S3 and substrate S1, respectively. 1X PBS standards are included in rightmost 3 lanes in each gel. All reactions were then electrophoresed in 20% polyacrylamide gels (6 μL/well loading) (C) Shows the ratio of post-shock to pre-shock fluorescence values observed for measurement of both enzymes in the blood and plasma of 4 individual rats. (D) Summarizes the mean values for results in (C), showing the p-values for a 2-tailed paired t-test between the pre- and post-shock values.

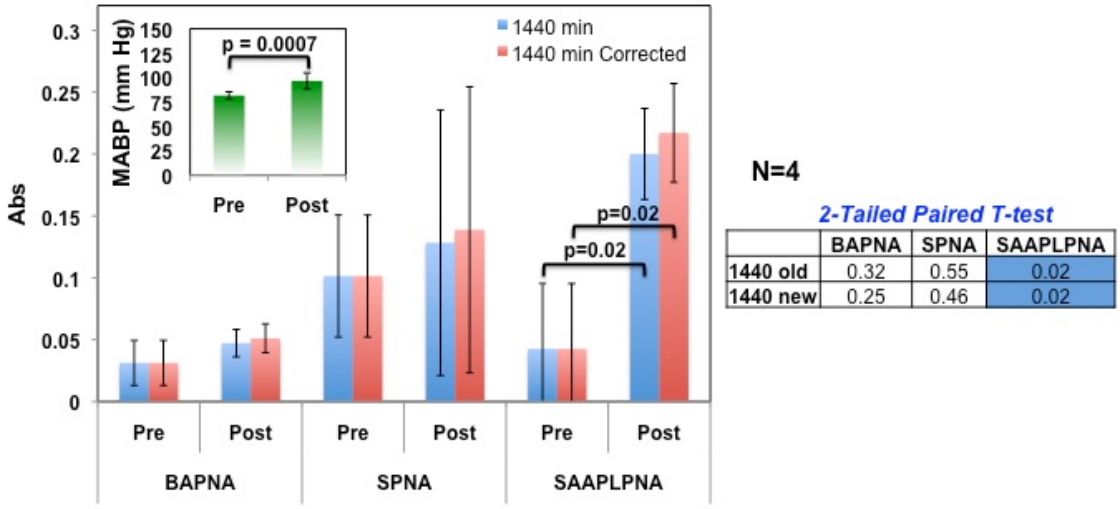


Figure 5.8: Corrected chromogenic substrate measurements in physiological shock (measurements by Dr. Alexander Penn). This is the same data as shown in Figure 5.6, but with a correction for the post-shock sample dilution with saline. Shows measured absorbance values of the t=90 minutes, t=180 minutes, and t=1440 minutes time points from chromogenic assays using BAPNA (measuring trypsin-like activity), SPNA (measuring chymotrypsin-like activity), and SAAPLPNA (measuring elastase-like activity) for both the pre- and post-shock heparin plasma samples. The table on the right summarizes 2-tailed paired t-tests comparing the pre- and post-shock chromogenic substrate measurements. The inset graph shows the mean arterial blood pressure (MABP) for both pre- and post-shock samples.

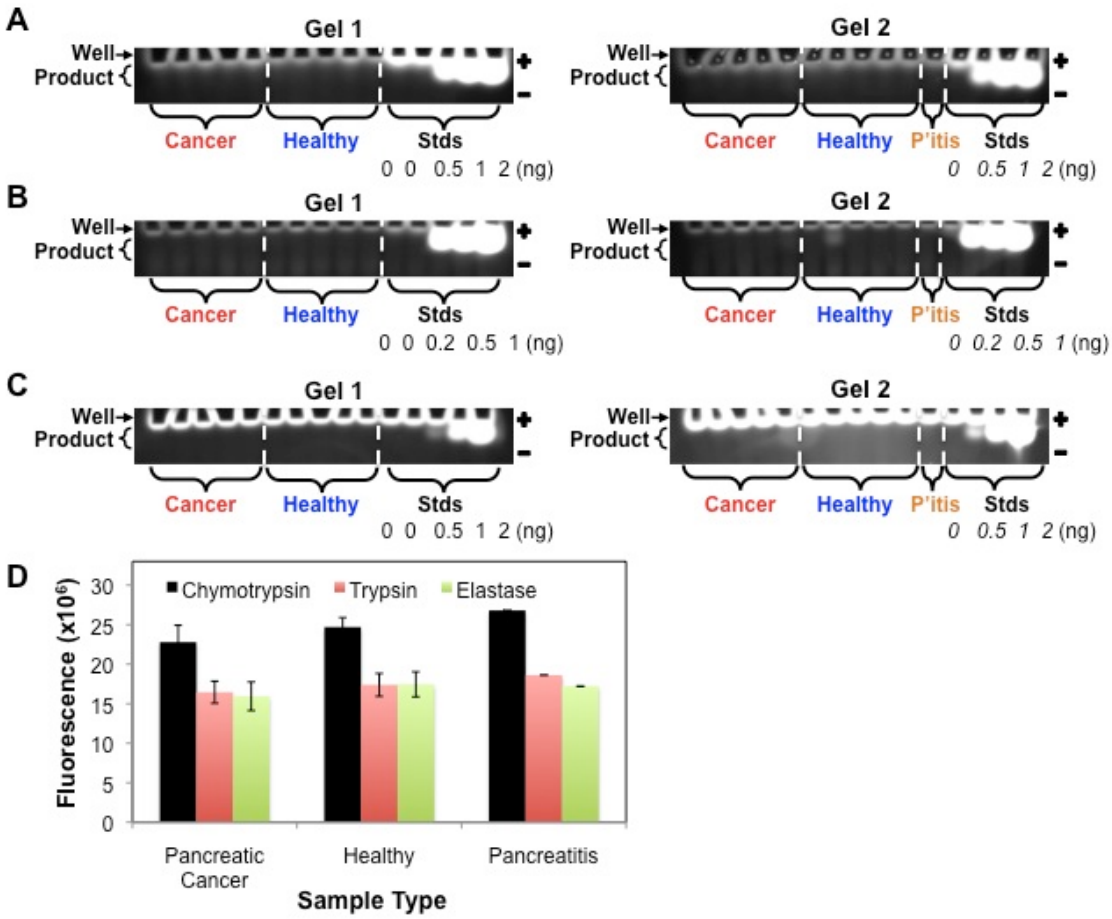


Figure 5.9: Pancreatic protease activity in pancreatic cancer. (A)-(C) Measurement of (A) chymotrypsin-like, (B) trypsin-like, and (C) elastase-like activity in EDTA plasma after 1 hour reactions with 0.5 mg/mL substrates S3, S1, and S2, respectively, and electrophoresis in 20% polyacrylamide gels (6 μL/well loading). The 10 pancreatic cancer samples (cancer), 10 normal healthy controls (healthy), and 1 pancreatitis sample (P'it is) are split amongst two gels (gel 1 and gel 2). Both gels also include standards in 1X PBS. (D) Summarizes the data of the 6 gels in (A)-(C), showing average fluorescence values for chymotrypsin-like activity (black), trypsin-like activity (red), and elastase-like activity (green).

Table 5.1: CV data for detection of MMP-2-like activity in type II diabetes

Sample Type	Between-Gel CV (%)
Std- 0 nM*/ MMP-2	25.6
Std- 1 nM*/ MMP-2	26.9
Std- 6 nM*/ MMP-2	14.7
Healthy Patient (Plasma)	36.1
Type II Diabetic (Plasma)	41.1

*0, 1, 6 nM = 0, 0.6, 2 ng (6 μL loading)

Table 5.2: CV data for detection of chymotrypsin- and trypsin-like activity in shock

Sample Type	Chymotrypsin		Trypsin	
	Within-Gel CV (%)	Between-Gel CV (%)	Within-Gel CV (%)	Between-Gel CV (%)
Std- 0 nM*	37.8	46.6	55.8	62.1
Std- 3 nM*	7.6	12.2	13.5	33.3
Std- 6 nM*	3.1	-----	2.7	-----
Pre-Shock (Blood)	11.9	-----	13.3	-----
Pre-Shock (Plasma)	15.9	-----	8.5	-----
Post-Shock (Blood)	7.0	-----	9.5	-----
Post-Shock (Plasma)	7.9	-----	12.7	-----

**0, 3, 6 nM = 0, 0.5, 1 ng (6 μL loading)*

Chapter 6:

Conclusions

6.1 Summary

As reviewed in Chapter 1, prior protease assays greatly depend on sample preparation, making them more costly, complex, time-consuming, laborious, and less accurate. This has generally limited their usefulness in biomedical research and has, perhaps worst of all, precluded their use in POC applications. The subject of this dissertation is an assay that has overcome this fundamental limitation, allowing the rapid measurement of *clinically relevant* levels of protease activity in microliters of unprocessed whole blood. More specifically, we have shown the following:

- In Chapter 2, this assay is first introduced through the development of two *fluorescence-labeled charge-changing peptide substrates*, one substrate for combined detection of chymotrypsin and trypsin and a second substrate for trypsin-specific detection. These negatively charged substrates produce a positively charged fluorescent cleavage fragment upon specific proteolysis by their target protease. After minimal electrophoresis, this cleavage fragment is then removed from whole blood, whose components are predominantly oppositely (negatively) charged.
- In Chapter 3, a straightforward improvement is introduced to this whole blood protease assay. In this novel approach, called *polyanionic focusing gel electrophoresis*, the positively charged cleavage fragment is concentrated during electrophoretic migration through a high-density gel doped with an oppositely charged (polyanionic) polymer,

poly-L-glutamic acid. This concentration occurs primarily due to electrostatic interaction between the cleavage fragment and the dopant. This technique, applied to measurement of trypsin activity, was shown to improve the LOD to clinically relevant levels in whole blood.

- In Chapter 4, the techniques developed in Chapters 2-3 are extended through the development of four new substrates for specific detection of elastase, chymotrypsin, MMP-2, and MMP-9. This shows that this versatile assay can be used for the rapid, clinically relevant detection of any protease in whole blood. It also reveals that the biggest challenge in further developing this protease assay is to design substrates that are sufficiently specific. While these initial attempts are modestly successful, it is obviously going to be very valuable for the development of novel diagnostics that even more specific substrates are developed.
- Finally, in Chapter 5, charge-changing substrates are used to measure protease activity in whole blood and plasma from animals and patients that have type II diabetes, pancreatic cancer, or that are in physiological shock. In that chapter, it is clearly shown that these sensitive substrates are able to differential normal from unhealthy samples, using minimal amounts of time (30 min. to 1 hour) and sample (6 μL). Further validation of these applications can potentially lead to several exciting opportunities: (a) a diagnostic test that can lead to making a treatment decision (MMP inhibitor) for type II diabetes, (b) the first ever rapid POC early-stage shock diagnostic, and (c) the first ever early diagnostic for deadly pancreatic cancer.

For the first time ever, novel protease diagnostic markers and therapeutic drug targets can be discovered and monitored through direct measurement of protease activity

in unprocessed blood samples. It is evident that this powerful whole blood protease assay makes possible new and *exciting opportunities* both in biomedical research and in the development of novel POC diagnostics.

6.2 Future Work

The whole blood protease assay developed in this dissertation is surprisingly straightforward, fast, robust, and sensitive. It is able to achieve clinically relevant detection in an hour or less, using simple electrophoretic gel formats and using microliters of unprocessed whole blood. However, it should be noted that there are *numerous possibilities* for greatly improving upon this assay in order to achieve even better specificity and sensitivity and even faster detection. There is also a simple way to make this assay more quantitative.

6.2.1 Improving Specificity

There are at least three approaches that can be used to develop more specific substrates (analytical specificity):

- First, the amino acid sequence composition (primary structure) can be optimized. This sequence determines the catalytic efficiency (k_{cat}/K_m) of substrate cleavage by both the target protease and by unintended targets (non-specific cleavage). Through the use of high-density combinatorial peptide arrays and high-throughput screening, optimum

sequences can be discovered that maximize cleavage by the intended protease target and that minimize cross-reactivity with unintended targets.

- In a second approach, the tertiary structure can be optimized to take advantage of the fact that the specificity of a protease is largely controlled by the "lock and key" interaction between a protease substrate and the active site of a protease. Structure-based rational design has been used in the development of novel inhibitors that specifically target MMPs involved in cancer progression (e.g. metastasis) [5, 177, 178]. Using a similar approach, rational design can be used to modify the tertiary structure of the substrate by the introduction of specific chemical moieties. These modifications would optimize the substrate's ability to better fit into and bind a protease's active site. Such an approach could be particularly useful in developing substrates for MMPs since many of them have overlapping primary structure specificities (common cleavage motif Pro-X-X-X_{Hy}). For example, substrates could be developed that are able to better fit into the active site of MMP-2 versus into MMP-9.
- Finally, the substrate specificity can be improved by using non-amino acid or non-natural amino acid sequences for the spacers outside of the cleavage sequence. Most of the charge-changing substrates developed in this dissertation have an amino acid sequence present simply to introduce space between the cleavage site and the fluorophore (e.g. Ala-Gly-Ala-Gly) and reduce steric hindrance. Also, they all have residues present such as Asp that are designed to make the substrate negatively charged prior to cleavage. Unfortunately, these additional amino acid sequences can introduce possible cleavage sites for non-target proteases (e.g. elastase). A simple solution to this issue can be used with the knowledge that all of the substrate sequences presented in this dissertation used

naturally occurring L-amino acids. Non-specific cleavage could be reduced through placing non-natural cleavage-resistant D-amino acids into the non-cleavage site portions of the substrate (e.g. spacers). This same concept was used by Roger Tsien's group in designing cleavage-resistant portions of activatable cell penetrating peptides [179].

It should also be emphasized that the improvements just discussed deal with analytical specificity. It can be argued that the importance of analytical specificity stems mostly from its affect, ultimately, on another type of specificity, clinical specificity. Analytical specificity determines how well a substrate can differentiate activity of one protease versus another, while clinical specificity determines how well this substrate can differentiate a diseased state from the healthy state. Even in the absence of perfectly analytical specificity (which has not be obtained for any protease assay), it is always feasible to improve clinical specificity through the use of multiplex detection. By measuring a signature of protease activity using multiple charge-changing substrates, there will be multiple independent markers that can be rapidly and simultaneously determined in a single unprocessed whole blood sample. Together, these independent markers can lead to more accurate disease diagnosis, as demonstrated by the multivariate analysis of 23 serum markers for pancreatic cancer by Hyakawa et al. [13].

6.2.2 Improving Sensitivity

There are several ways to improve sensitivity of the detection. The substrates presented in this dissertation used the organic fluorophore, Bodipy FL (Invitrogen). This provided a simple and robust means of labeling the substrates, but these labels were not

necessarily the most optimum choice possible. There are much brighter nanoparticle labels that can potentially be incorporated into this assay in order to achieve more sensitive detection:

- semiconductor (e.g. CdSe) quantum dots
- a tree of fluorophores conjugated to the ends of branches of a half-dendrimer (dendron)
- polymer-based fluorescent nanoparticles (e.g. polystyrene) or metal nanoparticles (e.g. gold or silver)
- metal quantum dots formed by the encapsulation of a metal in the interior of a dendron.

These labels not only provide a much brighter signal for detection, but can also improve the LOD because of their bulky size, which will make it easier to concentrate in electrophoretic gels (mobility is dependent on particle size). The challenge in using nanoparticle conjugates, however, will be in the ability to overcome several issues. First, many of these nanoparticle labels have large and random amounts of charge, making it difficult to design peptide sequences that will achieve the desired net charge change (negative to positive net charge upon proteolysis) upon fluorescent conjugation. Second, a major issue with nanoparticles is that they are heterogeneous in size and often aggregate. Individual substrate molecules may have random amounts of randomly sized labels and thus contribute random amounts of signal upon proteolysis. This is not as much of an issue for bulk detection of larger amounts of protease activity, but it is certainly more of a problem for achieving high-sensitivity detection of very low levels of protease activity. Finally, most nanoparticles have random and multiple numbers of

conjugation sites. Thus, an individual fluorescence-labeled substrate molecule may undergo a random amount of cleavage events before undergoing the desired charge change. For example, a quantum dot labeled with 4 peptide substrates may require all 4 to be cleaved before the overall net charge is positive. The random distributions of size, conjugation sizes, and charge are all issues that will need to be overcome in order to make it feasible to incorporate nanoparticle labeling into charge-changing substrates.

Another very simple avenue for further improving the LOD is to use thinner gel formats. Only two gel thicknesses were explored in this dissertation, 1-cm and 1-mm thick gels, with the thinner gels having at least an order of magnitude better LOD. Electrophoretic gel matrices introduce autofluorescent background. Thinner gels produce less of this background and allow more sensitive detection. Thus far, gels as thin as 50 μm have been produced [180]. Making the gels in this assay even thinner (micron-scale thickness) could feasibly result in an order of magnitude or better improvement in the LOD.

6.2.3 Improving Detection Speed

Primarily two steps control the speed of the entire protease detection process: (1) the time allowed for protease to react with substrate and (2) the electrophoresis time required to separate the fluorescent cleavage fragment from whole blood. Both of these can be dramatically reduced with straightforward optimization. As the LOD is improved, e.g. through gel miniaturization or the incorporation of more sensitive nanoparticle labeling, less reaction time will be required. In Chapter 3, the achieved LOD (0.3 ng/mL)

is 50- to 200-fold lower than the estimated reference levels (15-60 ng/mL) for trypsin. Thus, the reaction time (1 hour) could, by a rough estimate, be decreased by approximately 50- to 200-fold (18 seconds to 1.2 minutes). The speed can also be improved through higher voltage gradients, which are easier to achieve after gel miniaturization. Typical voltages for 8-cm long gels were 500V, producing a voltage gradient (electric field strength) of 62.5 V/cm. The velocity of a charged particle under an electric field, as explained in Chapter 1.4.1 (Equations 3-4), is directly proportional to this voltage gradient. Thus, the electrophoresis time can be reduced through the application of higher voltage gradients. Unfortunately, large format gels (e.g. 8-cm long gels), such as those that were used in this dissertation, will experience excessive Joule heating at higher voltage gradients. Capillary gels and micron-scale slab gels have a higher surface-to-volume ratio and are capable of withstanding much higher voltage gradients. For example, Heller et al. applied voltage gradients of up to 33 V/mm (330 V/cm) to 1-mm diameter capillaries during the separation of DNA fragments [130]. This gradient is approximately 5-fold higher than that used in the experiments of this dissertation. If such a voltage gradient could be applied to the protease assay, 10-minute electrophoresis time could potentially be as fast as 1-2 minutes. Thus, the combined improvements (miniaturization, nanoparticle labeling, higher voltage gradients) could bring the total assay time (currently > 1 hour) down to a couple of minutes or less. It should be re-emphasized that all of these improvements in speed, sensitivity, and specificity are for an assay already capable of measuring clinically relevant levels of protease activity in microliters of unprocessed whole blood.

6.2.4 Improving Quantification

Chapters 2-4 estimated the LODs of various enzymes by reacting known concentrations of enzyme with a fixed concentration of (uncleaved) substrate. Even though these assays are a direct measure of activity, the LODs were given in terms of a concentration (ng/mL or nM) or a load amount (ng), not in terms of activity. This is not ideal since a fixed amount of enzyme, obtained from a manufacturer, can have different activities depending on several conditions, for example: length of time stored in a freezer, number of freeze-thaw cycles, enzyme purity, and batch-to-batch variation. Activity is a more reliable form of quantification, and is more appropriate for an assay that measures protease activity. “Units” of activity are typically defined as the number of cleavage product molecules that are generated during a fixed amount of time. Unfortunately, in the experiments of this dissertation, it is not known what is the correlation between the fluorescence of the cleavage product band and the number of molecules of cleavage fragment that are contained in that band. In future experiments, activity units can be measured by using known concentrations of a “calibration standard”, which is the cleavage fragment obtained through chemical synthesis (not by proteolysis of the larger, intact substrate). Calibration standards would then allow the charge-changing substrate assay to quantify protease activity truly in terms of activity.

Chapter 5 provided comparisons of the relative differences in protease activity in normal and diseased samples without quantification in terms of a concentration or an activity. 1X PBS standards were often included in an attempt to help provide some estimate of concentration or activity in unknown blood and plasma samples, but they

really are not suitable, as currently designed, for a quantitative assay. These standards were obtained by reacting various concentrations of enzyme with a fixed concentration of substrate in 1X PBS. As stated above, such pseudo-standards cannot provide quantification in terms of activity units. This also does not provide a good estimate of protease concentration (or load). If the same amount of enzyme is spiked into buffer and into whole blood, there will be a different amount of cleavage fragment generated. This is due to endogenous co-factors, inhibitors, and proteases that are present in blood, but not in buffer. Peptide fragment calibration standards will not be affected by these differences. Using calibration standards, future sample testing experiments will be able to quantify the unknown protease activities that are detected in blood and plasma samples.

6.2.5 Further Clinical Testing

In the plasma, protease activity is curtailed by high concentrations (μM) of protease inhibitors such as α_1-antitrypsin and α_2-macroglobulin. Out-of-control, excessive protease activity may be a mechanism for the onset of many different medical conditions. Therefore, it would be useful to determine the ability of plasma protease inhibitors in buffering this excessive protease activity. This can be achieved through a kinetics study by reacting different concentrations of a charge-changing substrate ([S]) with a plasma sample that has been spiked with a fixed concentration of enzyme ($[E_t]$). The enzyme concentration would need to be sufficiently high in order to be detectable above endogenous protease activity levels. “No-enzyme” controls would allow for subtraction of endogenous protease activity (to obtain signal in RFUs). In-gel calibration

standards could be used to determine the correlation between the observed fluorescent signal and their corresponding reaction velocities (V_o). A pseudo-plasma (buffer or inhibitor deficient plasma) can be substituted for plasma to serve as a "no-inhibitor" control. From a double reciprocal plot of $1/V_o$ versus 1/[S], the Michaelis-Menten kinetics constants can be obtained for both the cases where inhibitors are present (normal plasma) or are absent (pseudo-plasma). The difference between these two can provide a measure of the protease inhibition. Using different individual pairs of spiked enzyme and substrate, this experiment can elucidate the plasma's ability to selectively block the activity of different proteases.

In addition to the study of plasma inhibition, there are also some fantastic opportunities for further clinical and pre-clinical testing for type II diabetes, physiological shock, and for pancreatic cancer:

- For type II diabetes, additional sample testing will be useful for further verifying the elevation of MMP-2-like activity. This testing should use glucose values as a selection criterion in order to make sure that the patients are categorized accurately (e.g., a "diabetic" is truly diabetic). Furthermore, testing with more specific substrates could potentially provide a greater ability to resolve diabetic and healthy samples. For example, the Chapter 4 substrates, S4 and S5, could be used to differentiate MMP-2 versus MMP-9 activity. Additional testing can also lend more support to using doxycycline as a potential new treatment option. Finally, future studies should test samples that are as fresh as possible in order to avoid the degrading affects of long-term storage (e.g. freezing and freeze-thaw cycles) (see Chapter 5.3.3).

- For physiological shock, additional sample testing of ~9-13 more rats could show that there is a statistically significant elevation of chymotrypsin-like and trypsin-like protease activity. Furthermore, as evident from the chromogenic substrate assays, it will be very useful to begin obtaining measurements of elastase-like activity as well. Using measurements of one or a combination of these three activities, future studies can estimate the clinical sensitivity and specificity of diagnosing physiological shock. Finally, the physiological shock experiments in this dissertation only used post-shock samples obtained 2 hours after injury (a hemorrhage). Future studies could therefore measure activities at different time points after injury (post-shock) in order to determine whether these markers are suitable for early diagnosis.
- For pancreatic cancer, future protease measurements should include fresh, whole blood and plasma samples that have not been treated with any chelator (e.g. EDTA). Such samples will have much more protease activity and should allow for a more accurate measurement. In these experiments, enough samples should be tested in order to discover statistically significant elevations of protease activity. It will also be important to determine the clinical sensitivity and sensitivity for the protease markers, either individually or in combination. Finally, using a genetic mouse model, measurements can be taken at different time points during the progression of pancreatic cancer. This is very important for establishing the diagnostic utility of protease markers in early diagnosis, particularly for pre-stage 1 pancreatic cancer.

In conclusion, there are several important points to remember for disease sample testing, in general. As shown by diabetes trials 1-3 (Chapters 5.3.1-5.3.2) and by the physiological shock trial (Chapter 5.3.4), there is more protease activity in whole blood

than in plasma. As implied by diabetes trial 4 (Chapter 5.3.3) and especially by the pancreatic cancer trial (Chapter 5.3.5), significant freezer storage time and treatment with chelators will degrade the protease activity in clinical samples. Thus, it is clear that it is best to use fresh, whole blood samples that have not been treated with any chelators. In such samples, protease activity is largest and determined most accurately (farther removed from background). Furthermore, some of the additional activity measured in blood, versus in plasma, may be non-disease related (e.g. non-specific cleavage by cell-membrane proteases). As evident in the physiological shock trial, this additional activity can reduce the difference between healthy and abnormal samples. Thus, while blood samples have more activity, it may be more beneficial in certain cases to obtain measurements in plasma instead. This issue can be ameliorated as the substrate specificity is improved enough in order to get sufficient accuracy for disease diagnosis using whole blood.

Appendix 1:

Derivation of Kinetics Equations

In our studies to determine the kinetic parameters of Ac-N-Asp-Gly-Asp-Ala-Gly-Tyr-Ala-Gly-Leu-Arg-Gly-Ala-Gly-diamino-ethyl-Bodipy FL, we used a first order analysis to directly estimate the k_{cat}/K_m ratio. The derivation that follows shows how this ratio was estimated using Michaelis-Menten kinetics.

Definition of variables:

v = the initial velocity of the reaction

V_{max} = the maximum velocity of the reaction

K_m = the Michaelis constant

k_{cat} = the turnover number

[S] = the concentration of substrate

$[S]_o$ = the initial concentration of substrate

[P] = the concentration of product

$[E_t]$ = the total concentration of enzyme (bound and unbound to substrate)

The 1st order rate constant for the proteolytic cleavage of the substrate by chymotrypsin and trypsin was obtained by using steady state Michaelis-Menten kinetics at a low substrate concentration. At [S] << Km, the Michaelis-Menten equation Eq. 1 reduces to Eq. 3, after substituting Eq. 2:

$$v = \frac{V_{\max}[S]}{K_m + [S]} \text{ [Eq. 1]}, \; k = \frac{V_{\max}}{K_m} \text{ [Eq. 2]}$$

$$v = \frac{V_{\max}[S]}{K_m + [S]} \approx \frac{V_{\max}}{K_m}[S] = k[S] \text{ [Eq. 3]}$$

Writing both sides of Eq. 3 in terms of [S] to form Eq. 4, rearranging to Eq. 5, and solving for [S], we obtain Eq. 6 and Eq. 7, the levels of substrate and product, respectively, over time:

$$-\frac{d[S]}{dt} = v = k[S] \text{ [Eq. 4]}$$

$$-\frac{d[S]/[S]}{dt} = k \text{ [Eq. 5]}$$

$$[S] = [S]_o e^{-kt} \text{ [Eq. 6]}$$

$$[P] = [S]_o - [S] = [S]_o - [S]_o e^{-kt} = [S]_o(1 - e^{-kt}) \text{ [Eq. 7]}$$

An alternative way to write Eq. 6 is as follows:

$$\frac{[S]}{[S]_o} = e^{-kt} \rightarrow \ln(\frac{[S]}{[S]_o}) = -kt \rightarrow \log_{10}(\frac{[S]}{[S]_o}) = -k * \log_{10}(e) * t$$

$$\rightarrow \log_{10}([S]) = -k * \log_{10}(e) * t + \log_{10}([S]_o) \text{ [Eq. 8]}$$

If the fluorescent emission is recorded from the beginning of the reaction until all of the substrate is converted to product, the fluorescent intensities can be related readily to the amount of product generated, [P](t), or substrate consumed, [S](t). Subsequently, a line can be fit to $\log_{10}([S])$ and the slope of that line, according to Eq. 8, will provide the linear rate constant. To compare the obtained linear rate constant with those of other substrates in the literature, when the literature reports k_{cat}/K_m rather then k, we can obtain estimates of k_{cat}/K_m for our substrate by the following derivation:

$$V_{\max} = k_{cat}[E_t] \quad \text{[Eq. 9]}$$

$$k = \frac{V_{\max}}{K_m} = \frac{k_{cat}[E_t]}{K_m} \quad \text{[Eq. 10]}$$

$$\therefore \frac{k_{cat}}{K_m} = \frac{k}{[E_t]} \quad \text{[Eq. 11]}$$

Appendix 2:

Derivation of Correction Factor for Physiological Shock Trial

In the physiological shock trial, 2 mL saline is perfused into the animal in order to recover lost circulation volume during blood sample collection. Thus, it is necessary to account for this dilution of the plasma, which will affect the detected level of protease activity for post-shock samples.

Definition of variables:

W = weight of the rat

BV_i = initial volume of blood in the circulation

PV_i = initial volume of plasma in the circulation

PV_f = final volume of plasma in the circulation

DF = dilution factor

F = uncorrected fluorescent signal

F_c = corrected fluorescent signal

The initial amount of blood in the circulation, prior to sample collection, can be estimated from the weight of the rat by the following formula:

$BV_i = W*0.06$ [Eq. 1]

If we estimate that approximately 50% of the blood volume is plasma, then multiplying Eq. 1 by 0.5 provides the approximate volume of plasma in the circulation:

$$PV_i = (W*0.06)*0.5 = W*0.03 \text{ [Eq. 2]}$$

If approximately 2 mL of blood is removed from the animal during sample collection, then it has effectively lost approximately 1 mL of plasma. After returning 2 mL of saline to the animal, the total volume of plasma is increased by 2 mL since cells are not being returned. Eq. 3 thus provides the final volume of plasma in the circulation:

$$PV_f = W*0.03 - 1 + 2 = W*0.03 + 1 \text{ [Eq. 3]}$$

The ratio PV_f/PV_i provides the dilution factor, DF, which describes how much the plasma has been diluted by saline:

$$DF = PV_f/PV_i = (W*0.03 + 1)/ (W*0.03) = 1 + (100/(3*W)) \text{ [Eq. 4]}$$

The original fluorescent signal, F, from a post-shock sample can then be corrected using the following formula:

$$F_c = F*DF \text{ [Eq. 5]}$$

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