Characterization of a Secreted *Escherichia coli* O86a: K61 Protease that Inactivates Human Coagulation Factor V

by

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Abstract

Background: *Escherichia coli* (*E.coli*) O86a:K61 belongs to the Enteropathogenic *E. coli* (EPEC) group of pathogens. Acute gastroenteritis affects 2-4 billion people annually and EPEC is associated with 10-40% of hospitalized diarrhea cases globally. Coagulation Factor (F) V circulates as an inactive procofactor (Mr 330kDa) which upon thrombin activation to the active cofactor, FVa, functions in *prothombinase* to accelerate prothrombin to thrombin conversion by 300,000-fold. The ability of *E.coli* O86a:K61 to cause intestinal hemorrhage is of interest because previous research demonstrated that during *E.coli* O86a:K61 sepsis in baboons, a dose-dependent inactivation of FV was observed as the bacterial dose increased. These results suggested a secreted *E.coli* protease may have mediated this effect on FV. This research has focused on the purification, identification, and characterization of a secreted *E. coli* O86a:K61 protease that inactivates FV. The final partially-purified protease inactivated FV to a 250kDa product by immunoblotting, and possessed a 900-fold increase in specific activity versus FV in human plasma compared to the culture supernatant. At least 3 proteins were observed upon SDS-PAGE. Proteolytic inactivation of FV was activated by up to 500-fold with β-mercaptoethanol and 2-fold with 1M urea. The protease was heat stable retaining all of its activity versus FV after 1h at 70°C or 80°C, and partial activity (50%) at 95°C. Proteolysis of FV was blocked by 90% with alpha-1-protease inhibitor; however, the protease was resistant to 1.5 mM PMSF, and unaffected by E64, or iodoacetamide. FV is a major regulator of the coagulation process and its inactivation by the secreted *E.coli* protease would be expected to result in a net bleeding tendency which may contribute to the mucosal hemorrhage observed in humans with associated hemorrhagic colitis. Proteolytic inactivation of FV is predicted to result in decreased bacterial containment by host fibrin thereby increasing pathogen survival and growth. FV inactivation by the secreted *E.coli* protease may be part of a novel pathogenic virulence mechanism that deregulates the blood coagulation process to enhance bacterial infectivity and transmission.

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List of Abbreviations

A1PI Alpha 1-Protease Inhibitor

APPT Activated Partial Thromboplastin Time

APC Activated Protein C

AT Antithrombin

BCA Bicinchoninic Acid

BSA Bovine Serium Albumin

CBB Coomassie Brilliant Blue

CFF Centrifugal Flow Filtration

cfu Colony Forming Units

CHAPS 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate

Cmc Critical Micelle Forming Concentration

DIC Disseminated Intravascular Coagulation

DTT Dithiothreitol

E. coli *Escherichia coli*

E64 N-[N-(L-3-transcarboxyirane-2-carbonyl)-L-Leucyl]-agmatine

ECL Enhanced Chemiluminescence

EDTA Ethylenediaminetetraacetic Acid

EHEC Enterohaemorrhagic *Escherichia coli*

EPEC Enteropathogenic *Escherichia coli*

EspC Enteropathogenic *Escherichia coli* protease C

EspP Extracellular Serine Protease Plasmid-Encoded

ETEC Enteropathogenic *Escherichia coli*

ExPEC Extraintestinal Pathogenic *Escherichia coli*

F Factor

FDPs Fibrinogen Degradation Products

HBS Hepes Buffer Saline

Hbp Hemoglobin Protease

HMWK High Molecular Weight Kininogen

IgG Immunoglobulin G

IL Interleukin

kDa Kilodalton

Kgp Lysine Gingipain

LB Luria-Bertani Broth

LD Loading Dye

LPS Lipopolysaccharide

MA Minimal A Media

MWCO Molecular Weight Cut Off

NHP Normal Human Pooled Reference Plasma

OmpC Outer Membrane Protein C

OmpF Outer Membrane Protein F

OmpN Outer Membrane Protein N

OmpT Outer Membrane Protein T

OMVs Outer Membrane Vesicles

PBS Phosphate Buffer Saline

PBS_{TW} Phosphate Buffer Saline Tween-20

Pet Plasmid-Encoded Toxin

Pic Protease Involved in Intestinal Colonization

PK Plasma Kallikrein

Pla Plasminogen Activator

PMSF Phenymethanesulfonyfluoride

PT Prothrombin Time

PVDF Polyvinylidene Fluoride

RgpA Arginine Gingipain A

RgpB Arginine Gigipain B

Sat Secreted Autotransporter Toxin

SDS Sodium Dodecyl Sulfate

SDS-PAGE Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SM Starting Material

SPATE Serine Protease Autotransporters of *Enterobacteriaceae*

spp Species

STEC Shiga Toxin Producing *Escherichia coli*

TEM Transmission Electron Microscope

TF Tissue Factor

TFF Tangential Flow Filtration

TFPI Tissue Factor Pathway Inhibitor

TM Thrombomodulin

TNF Tumour Necrosis Factor

tPA Tissue Plasminogen Activator

uPA Urokinase-Type Plasminogen Activator

UPEC Uropathogenic *Escherichia coli*

Introduction

The Coagulation System

The primary function of the coagulation system is to prevent blood loss in response to injury or trauma. The coagulation system is characterized by a number of unusually large zymogens and procofactors that require serine protease activation for sequential, rapid, highly localized production of an insoluble fibrin clot. The coagulation cascade may be initiated by one of two pathways: the contact (Intrinsic) pathway, or the tissue factor (Extrinsic) pathway. Activation of either pathway results in the activation of the Common pathway, and the generation of thrombin, the master serine protease regulator of the coagulation cascade, and the enzyme that converts soluble fibrinogen into an insoluble fibrin clot.

Activation of the contact pathway occurs after damage to endothelium lining of the blood vessel. Factor (F)XII is activated after coming into contact with the electronegative surface of damaged endothelial and sub-endothelial cells. The active serine protease, FXIIa, then activates FXI and prekallikrein, to FXIa and kallikrein, respectively. These proteins are anchored to the vessel wall by high molecular weight kininogen (HMWK). Kallikrein serves to further activate neighbouring FXII proteins in a positive feedback loop, whereas FXIa, when in complex with its thrombin activated cofactor VIIIa (*Intrinsic tenase*), proteolytically activates FX into FXa. Activation of FX marks the end of the Contact activation pathway, and the beginning of the Common pathway.

The tissue factor pathway is considered the most essential pathway for the production of a fibrin clot. This notion is based on the observation that individuals deficient in contact factors, FXII, FXI, kininogen, or kallikrein do not suffer from bleeding disorders (Colman and Schmaier 1997). As its name implies, activation of the Extrinsic pathway requires activation by tissue factor (TF), a protein which is sequestered away from the bloodstream in the non-injured/ normal state. Upon vessel

Figure 1 The Blood Coagulation Cascade. The coagulation cascade is divided into an Intrinsic, Extrinsic, and a Common pathway. Coagulation factors of the Entrinsic pathway are represented in black, coagulation factors of the Extrinsic pathway are represented in purple, coagulation factors of the Common pathway are represented in green, and are designated by an "a" upon activation. Arrows symbolize the proteolytic conversion within the respective pathway. Factors in red are inhibitors and red arrows symbolize inactivation events. Proteins that participate in the fibrinolysis system are represented in orange. The following abbreviations are used: F, factor; HK, high molecular weight kininogen; PK, plasma kallikrein; TFPI, tissue factor pathway inhibitor; tPA, tissue plasminogen activator; and uPA, urokinase-type plasminogen activator. Modified from Tapper and Herwald (2000).

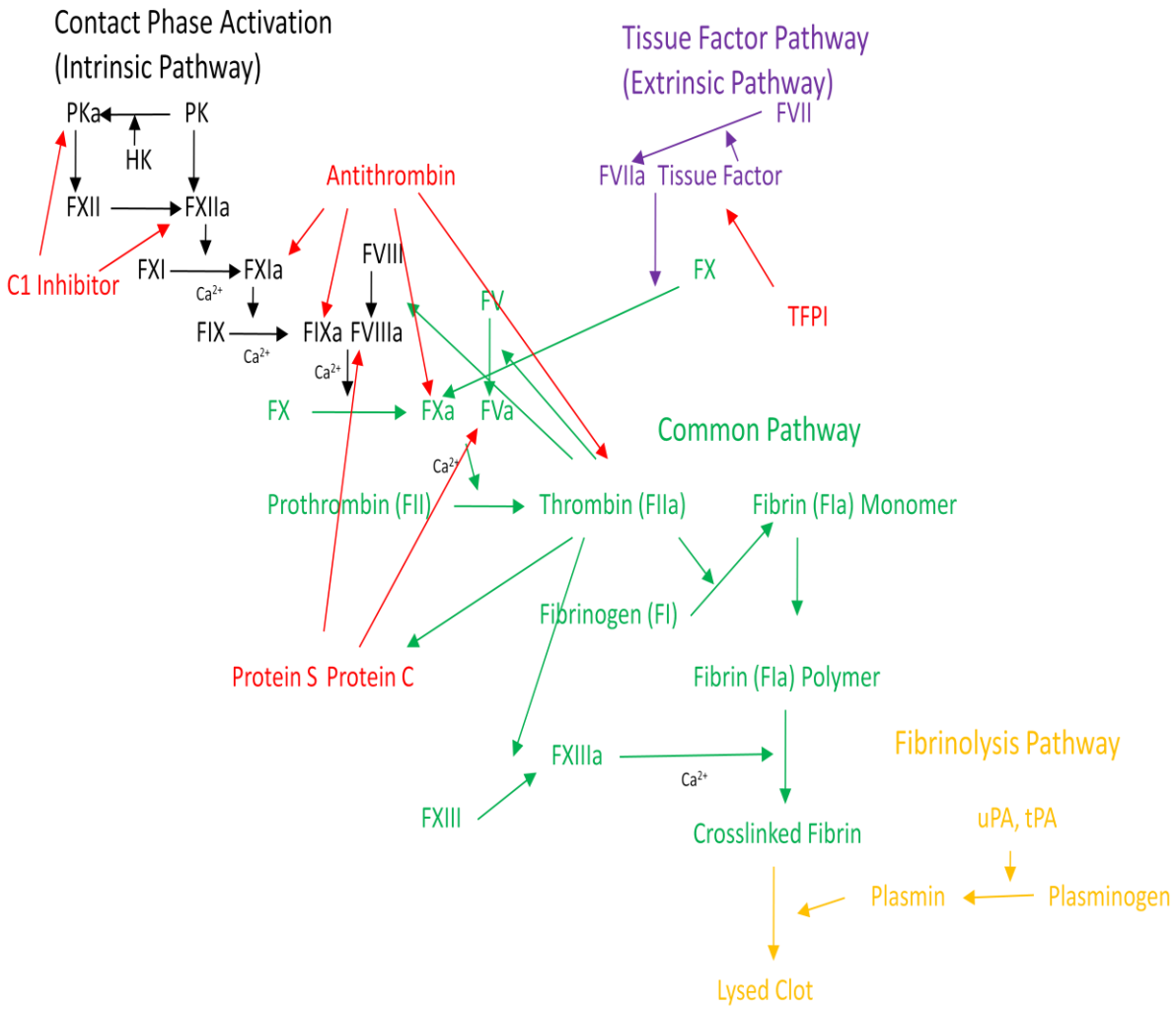

Contact Phase Activation
(Intrinsic Pathway)
Tissue Factor Pathway
(Extrinsic Pathway)
PKa
PK
HK
FXII
FXIIa
FVII
FVIIa
Tissue Factor
Antithrombin
C1 Inhibitor
FXI
FXIa
Ca2+
FVIII
FIX
FIXa
FVIIIa
Ca2+
Ca2+
FX
TFPI
FV
FX
FXa
FVa
Common Pathway
Ca2+
Prothrombin (FII)
Thrombin (FIIa)
Fibrin (Fla) Monomer
Fibrinogen (FI)
Protein S
Protein C
Fibrin (Fla) Polymer
FXIIIa
Ca2+
Fibrinolysis Pathway
FXIII
Crosslinked Fibrin
uPA, tPA
Plasmin
Plasminogen
Lysed Clot

Figure 1.

damage, TF is exposed to the bloodstream on the cell membrane of subendothelial cells, platelets, and leukocytes. When TF is exposed to the blood, it complexes and activates the serine protease FVII to FVIIa. The TF-FVIIa complex is then capable of activating FIX (Contact pathway) and FX, initiating the Common pathway.

The Common pathway begins with the generation of the active serine protease FXa. Initially FXa alone is capable of converting small amounts of prothrombin (FII) to thrombin (FIIa). Thrombin is then capable of activating the procofactor FV to FVa, allowing it to complex with FXa, calcium, and an anionic membrane (*Prothrombinase complex*), effectively increasing the rate of conversion of prothrombin to thrombin by approximately 300,000-fold (Krishnaswamy et al 1987). Sufficient activation of prothrombin to thrombin inevitably results in the generation of a fibrin clot. Thrombin is also capable of activating several proteins upstream in the cascade, resulting in a positive feedback loop. Thrombin proteolytically activates FXI, FIX, FVIII, and FV, resulting in the generation of additional thrombin. Thrombin also proteolytically activates fibrinogen (FI) to its insoluble and self polymerizing form, fibrin (FIa). The transglutaminase FXIII is also activated by thrombin to FXIIIa. FXIIIa crosslinks and stabilizes the developing fibrin clot, and prevents the clot's proteolytic degradation.

Regulation of the Coagulation Cascade

Activation of the coagulation cascade must be rapid, and highly localized to prevent blood loss; however, the system must be tightly regulated to prevent unwanted fibrin deposition. The coagulation system is regulated at several levels of the cascade. Thrombin, the master regulator of the cascade, is essential to the production of fibrin; however, it also functions in preventing excessive clot formation by binding to thrombomodulin (TM), a membrane bound protein of the endothelium. When thrombin is bound to TM, it is converted from a procoagulant enzyme to an anticoagulant enzyme. The thrombin/TM complex is not capable of activating fibrinogen, but instead rapidly activates the

anticoagulant protease protein C to activated protein C (APC). APC, in combination with its cofactor Protein S, inhibits further generation of thrombin by proteolytically inactivating FVa and FVIIIa, which are the essential cofactors for the *Prothrombinase* and *Intrinsic tenase* complexes, respectively. The circulating protease tissue factor pathway inhibitor (TFPI) ensures that the developing fibrin clot does not spread to unwanted areas of the circulatory system by inhibiting the TF/VIIa complex, and FXa (Jesty et al 1996). Antithrombin (AT) is also a potent inhibitor of fibrin formation by inhibiting thrombin directly in a 1:1 ratio, a process which is catalyzed by heparin (Rosenberg and Damus, 1973). AT also inactivates FXa, FIXa, FXIa, and to some extent the TF/FVIIa complex (Carlson et al 1985). These inhibitors of coagulation are necessary to allow for normal hemostasis.

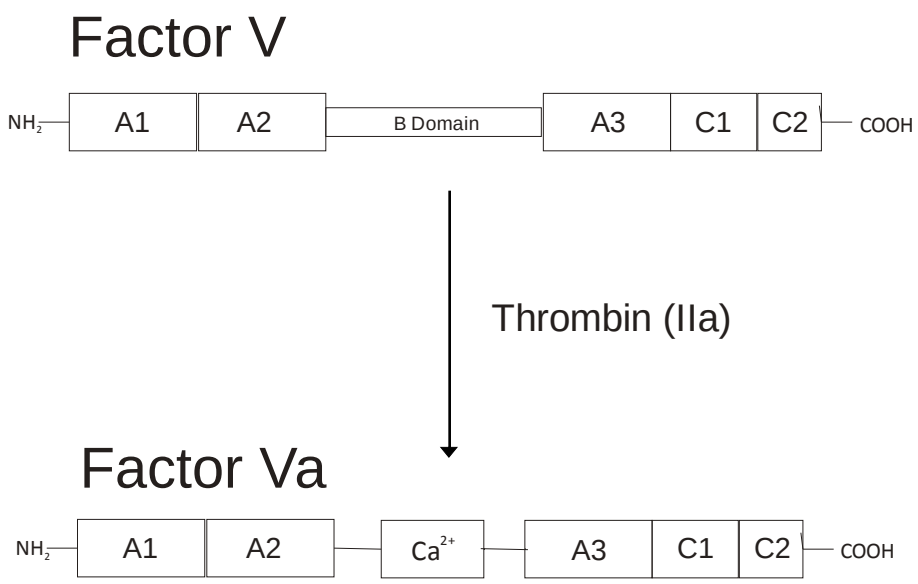
After vessel injury and subsequent fibrin formation, the fibrin clot must be broken down as tissues are repaired and the wound is healed. It is the role of the fibrinolysis system to degrade fibrin when it is no longer needed. Plasminogen is a circulating zymogen which is activated by tissue plasminogen activator (tPA) or by urokinase-type plasminogen activator (uPA) to plasmin, directly on the fibrin surface. The serine protease plasmin is responsible for the degradation and solubilisation of the fibrin clot to fibrinogen degradation products (FDPs). The coagulation system and the fibrinolytic system are tightly regulated to maintain hemostasis. Over-activation of the coagulation system or under-activation of the fibrinolytic system results in thrombosis (Excessive clot formation), whereas under-activation of the coagulation system or over-activation of the fibrinolytic system results in bleeding tendencies.

Factor V

Factor V (FV) is a key regulator of thrombin generation, and thus the overall coagulation process. Individuals who are deficient of functional FV (Parahemophilia) present with bleeding tendencies in the skin (44%), mucosa (44%), joints (23%), muscles (23%), genitourinary tract (19%),

Figure 2 Factor V Activation and Participation in the *Prothrombinase* Complex. A diagram of the organization of the human factor V molecule with domain structure is depicted in Panel A. Activation of FV by thrombin to the active procofactor, FVa, is the result of 3 sequential cleavages in the B-domain, which then dissociates. Following activation, the active cofactor is a heterodimer composed of a heavy chain, a divalent cation and an associated light chain. Once activated by thrombin, FVa is capable of participating in the *Prothrombinase* complex (Panel B). FVa in combination with a phospholipid membrane, calcium, and the serine protease FXa, can then proteolytically activate prothrombin to thrombin. Modified from Mann and Kalafatis (2003).

A)


Factor V
NH2
A1
A2
B Domain
A3
C1
C2
COOH
Thrombin (IIa)
Factor Va
NH2
A1
A2
Ca2+
A3
C1
C2
COOH

B)

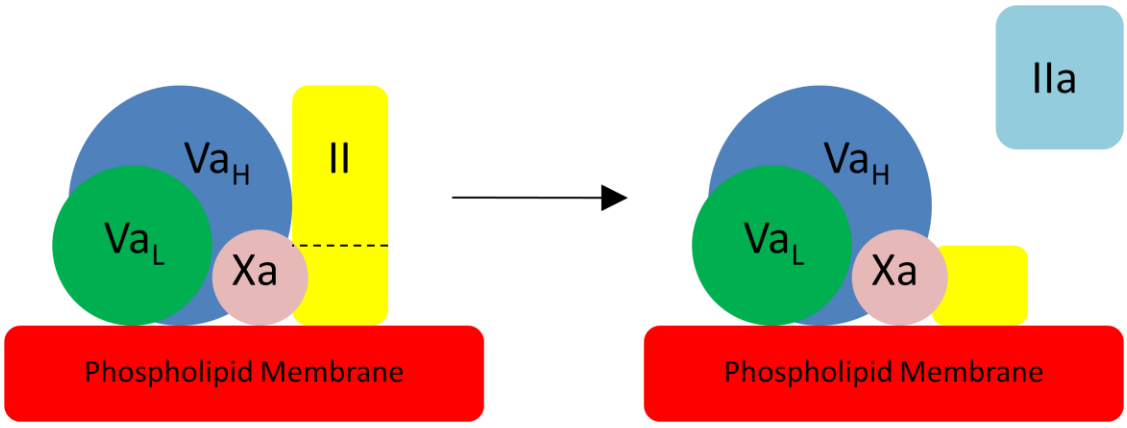

VaH
II
VaL
Xa
Phospholipid Membrane
IIa
VaH
VaL
Xa
Phospholipid Membrane

Figure 2.

gastrointestinal tract (6%), and the central nervous system (8%) (Huang and Koerper 2008). FV was discovered by Owren in 1943 (Owren and Cooper 1955), and first purified to homogeneity in 1979 by Nesheim (Nesheim et al 1979). FV circulates in human plasma at approximately 20-40nm as a 330,000kDa procofactor, with a domain structure of A1 A2 B A3 C1 C2. In addition, approximately 20% of the total human FV pool is present in platelet α-granules. The 2196 amino acid procofactor is synthesized primarily in the liver; however, megakaryoctes are also capable of synthesizing FV. FV is activated *in vivo* by 3 sequential cleavages in the B-domain by thrombin at Arg^{709}, Arg^{1018}, and Arg^{1545} (Orfeo et al 2004). The B domain then dissociates from the active FVa molecule, which can then bind FXa on a phospholipid membrane surface to form the P*rothrombinase* complex. This complex is the essential activator of prothrombin, as it increases the conversion of prothrombin to thrombin by 300,000-fold, compared to FXa acting alone. APC is the primary physiological regulator of FVa. Protein C is activated by thrombin, when coupled with the cofactor TM as part of a negative feedback loop. APC inactivates FVa by competing for binding with FXa; once in complex with FVa, APC proteolytically cleaves FVa at residues Arg^{506}, Arg^{306}, and Arg^{679} of the heavy chain. Individuals with the FV^{LEIDEN} allele generate a FVa molecule which is resistant to inactivation by APC. FV^{LEIDEN} represents the most common genetic cause of venous thrombosis in humans (Dahlback 2003); therefore, FV activation and inactivation are essential to maintaining normal hemostasis.

The Coagulation System and the Innate Immune Response

The coagulation systems and the immune system are interconnected and the most striking evidence of the interplay between the two systems comes from the horseshoe crab which has a completely integrated system that evolved nearly 500 million years ago and remains unchanged today (Delvaeye and Conway 2009). The coagulation system provides the host with a means to quarantine and sequester the infectious agent. The coagulation system is activated in general proximity to, or directly

on the bacterial cell membrane (Loof et al 2011) effectively preventing pathogen dissemination throughout the host and allowing for a localized immune response. The generation of *Prothrombinase*, and subsequently thrombin, is essential to preventing bacterial infection.

Thrombin generation in response to bacterial infection induces the release of proinflammatory cytokines from endothelial cells, mural cells, epithelial cells, adipocytes, and immune cells (Drake et al 1992; Fujita et al 2008; Wadgonkar et al 2008). Thrombin acts as a chemotactic factor for monocytes and neutrophils, and it is capable of directly activating complement component 5 (Huber-Lang et al 2006). Degradation of fibrin also stimulates the inflammatory response through activation of cytokines and chemokines such as TNF-α, IL-β, and macrophage chemotactic protein-1 (Szaba and Smiley, 2002). These inflammatory cytokines and chemokines also activate the coagulation system. Without proper regulation the inflammatory response enhances the coagulation process; that in turn, enhances the inflammation response, this process may cycle out of control and result in severe pathological conditions such as disseminated intravascular coagulation (DIC), which is associated with extremely high mortality rates (Esmon, 2005).

Bacteria and the Coagulation System

It is not surprising that bacteria have evolved mechanisms to subvert the coagulation system during infection. The coagulation system, like the immune system, may be deregulated by an invading bacterium to enhance their growth and survival. Several virulence factors have been identified which are capable of either activating or inactivating the coagulation system depending on which is more advantageous to the pathogen. It is theorized that during early stages of infection it may be advantageous to the pathogen and the host to initiate coagulation. This allows for the generation of a fibrin “shield” around the pathogen, which provides protection against phagocytosis by monocytes/macrophages, and allows for proliferation while preventing dissemination throughout the

host. However, once critical numbers are reached the bacteria may degrade the surrounding fibrin clot and disseminate throughout the host, and to new hosts. Others theorize that the activation of the coagulation system allows for the generation of a fibrin clot at the site of infection providing a localized immune response and containment for the host, and that bacterium have evolved means to prevent this from occurring. Either of these views requires the eventual breakdown of the clot to allow for dissemination of the pathogen. Both *Escherichia coli (E. coli)* and *Salmonella* spp express cell surface organelles which are capable of binding and depleting coagulation factors, which promote a hypocoagulatory state in mice (Herwald et al 1998).

Secreted Bacterial Proteases and the Coagulation System

Bacterial extracellular proteases from different pathogens provide resistance against the host immune defence system. Proteases from a variety of pathogens are essential virulence factors during infection. They may act to degrade antibodies (Molla et al 1988), complement proteins (Oda et al 1990), and may increase vascular permeability (Kaminishi et al 1990). Bacterial proteases may also deregulate the coagulation system, or directly degrade fibrin (Imamura et al 1995). There are two ways this may be accomplished: activation of host proteins which normally regulate coagulation or direct inactivation of coagulation proteins. Plasminogen activator (Pla), a membrane bound protease of *Yersinia pestis* which is essential to pathogenicity (Suomalainen et al 2007) activates the human zymogen plasminogen to its active and potent anti-coagulant form, plasmin. The protease is also capable of inactivating the main circulating inhibitor of plasmin α-2-antiplasmin. Arginine gingipain A (RgpA), Arginine gingipain B (RgpB), and Lysine gingipain (Kgp) are secreted/vesicle-bound cysteine proteases of *Porphyromonas gingivalis* which are capable of directly degrading fibrinogen in human plasma (Imamura et al 1995). EspP (Extracellular serine protease plasmid-encoded) is a secreted protease of *E. coli* O157: H7 that is capable of directly cleaving human coagulation FV (Brunder et al 1997).

Outer Membrane Vesicles

Secreted proteins of several pathogenic bacteria are critical to establishing colonization and virulence within their hosts. In addition to secreted virulence mechanisms, outer membrane vesicles (OMVs) of gram-negative bacteria may be implemented to establish a colonization niche. OMVs are produced via blebbing of gram-negative bacterial outer membranes and have been frequently regarded as cell debris or microscopy artefacts (Kulp and Kuehn 2010). No inner membrane or cytosolic components are incorporated into OMVs, suggesting that the blebbing of the outer membrane occurs in an organized fashion; however, little is known about the mechanism of OMV formation (Haurat et al 2010). OMVs are produced ubiquitously under the appropriate growth conditions from both pathogenic and non-pathogenic bacteria. OMVs may be used to facilitate horizontal gene transfer as they readily take up exogenous DNA; they are used in cell defence during phage infection, and to concentrate enzyme/enzyme complexes in their environment without expending energy in moving the cell. OMVs provide a protective function, allowing for transport of otherwise labile components (Such as protease-susceptible peptides) through the environment (Kesty and Kuehn 2004). OMVs from pathogenic bacteria are rich in lipopolysaccharide (LPS) and are associated with a variety of virulence factors such as: proteases, adhesins, hemolysins, toxins (Bomberger et al 2009; Haurat et al 2010) and have the ability to absorb host antibodies (Kulp and Kuehn 2010). These so-called “bacterial bombs” attack host tissues, deregulate host defences, and are involved in the hijacking of host cell processes (Mashburn-Warren and Whitely, 2006) which has resulted in intensive research over the past 5-10 years. Several pathogenic *E. coli* have been shown to secrete OMVs containing toxins such as heat-labile enterotoxin (Kuehn and Kesty 2005), Shiga toxin, and Cytolysin A (Yokoyama et al 2000). Injection of only 25µg of OMVs from intestinally derived *E. coli* resulted in systemic inflammatory response syndrome and death after 36h in mice (Park et al 2010). However, no OMV proteases have been reported to interact with the coagulation

system. Proteases have not been reported to associate with *E. coli* OMVs, nor have OMVs been reported with Enteropathogenic *E. coli* (EPEC) strains such as *E. coli* O86a: K61.

Bacteria and Factor V

The procofactor FV is essential to maintaining normal hemostasis. This is most clearly demonstrated in mice where FV deficiency is associated with a completely lethal phenotype during early embryonic development (Cui et al 1996). Curiously, only 0.1% of normal FV levels are required to rescue the lethal phenotype in mice (Mann 2000). Platelet or plasma derived FV levels have a direct effect on survival in *Streptococci*-induced sepsis models in transgenic mice (Sun et al 2009). In Sun et al (2009); transgenic mice were generated which had 45% or 15% plasma derived FV with no platelet pool, or 3% normal platelet FV levels with no plasma derived FV. Interestingly, significant differences in mortality rates were observed in each group, and compared to controls which had normal FV levels after experimentally induced sepsis with *Streptococcus pyogenes*. Sun et al (2009) clearly demonstrated the importance of FV during bacterial infection, and their results possibly explain the necessity of generating 1000-fold more FV than is necessary to maintain the coagulation health of the host. FV inactivation during bacterial infection would therefore be expected to increase morbidity/mortality in infected patients, by promoting a net bleeding tendency. During experimental *E. coli* O86a:K61 induced sepsis in baboons, a dose dependant relationship of FV inactivation was observed with increasing doses of infused bacteria (Samis et al 2009). Infusion of baboons with 2.40×10^6 (Low), 1.05×10^8 (Medium), or 6.49×10^8 (Lethal) cfu kg^{-1} of *E. coli* O86a:K61 resulted in the reduction of FV activity to 80%, 50%, or 20% of pre-infusion levels, respectively. Also, inactivation of FV was associated with the generation of a 250kDa cleavage product. These results suggest that a secreted *E. coli* protease may have mediated the cleavage and inactivation of baboon FV. FV inactivation may be essential to the establishment of *E. coli*

infection, as several strains are capable of producing proteases which degrade FV directly; or indirectly via activation of host proteases.

The high prevalence of the FV^{LEIDEN} allele (4.4% of Caucasians in America) (Gregg et al 1998) which encodes a FV protein that is resistant to inactivation by the host protease APC, and is associated with thrombosis in carriers, also speaks to the importance of FV activity during infection. One would expect heavy selective pressure to remove the FV^{LEIDEN} allele from the human population; however, FV^{LEIDEN} is thought to infer resistance to infection, and this has been demonstrated in LPS challenged mice (Weiler et al 2004). It is not surprising that FV is a target of several virulence factors during infection: EspP, EspC (Enteropathogenic *E. coli* protease C), Pet (Plasmid-encoded toxin), Pic (Protease involved in intestinal colonization), and Sat (Secreted autotransporter toxin), which belong to the family of SPATEs (Serine protease autotransporters of *Enterobacteriaceae*) and are all capable of cleaving FV. These proteases are capable of directly cleaving FV in human plasma, or FV that is added to serum, however, the effects on FV functional coagulation activity were not investigated (Brunder et al 1997). Aside from the SPATEs, several *E. coli* strains also produce OmpT (Outer membrane protein T), a membrane bound protease which is capable of activating the host fibrinolytic protease plasminogen to plasmin (Sodeinde and Goquen 1989). The primary role of plasmin is to degrade fibrin; however, it also proteolytically inactivates FVa and FVIIIa. Therefore, FV is an important protein during bacterial pathogenesis and a virulence target during *E. coli* infections.

Study Rationale

E. coli is responsible rational for numerous human and animal diseases including: diarrhea, hemorrhagic colitis, haemolytic-uremic syndrome, urinary tract infection, systemic inflammatory response syndrome, and sepsis (Johnson et al 2005; Johnson and Russo 2001; Chen and Frankel 2005; Laupland et al 2008; Croxen and Finlay 2010; Park et al 2010). It has been estimated that *E. coli* related

infections cost the US healthcare system approximately 1.1-2.8 billion dollars annually and accounted for approximately 40,000 deaths in 2001 alone (Russo and Johnson 2003). Traditionally, *E. coli* infections have not commanded attention due to the susceptibility of *E. coli* to antimicrobial therapy; however, antibiotic resistance amongst *E. coli* clinical isolates has been steadily increasing (Gupta et al 2001). Due to the emergence and increasing prevalence of antimicrobial resistance, new therapeutic approaches are required. Targeting virulence factors as an alternative approach to antimicrobial therapy is promising because it may allow clinicians to prevent pathogenesis without putting heavy selective pressures on the pathogen to evolve resistance (Cegelski et al 2008). Since it has been previously demonstrated that FV is not only a critical component of the host's coagulation system, but also of the immune response during bacterial infection, the goal of this study was to characterize and identify a secreted *E. coli* protease which is capable of cleaving and inactivating human FV in human plasma.

Hypothesis

Factor V is inactivated by an unknown secreted protease from *E. coli* O86a:K61 as part of a novel virulence mechanism that deregulates the coagulation process. Proteolytic cleavage and inactivation of FV would be expected to cause a net bleeding tendency in *E. coli* infected hosts.

Study Objectives

i) Develop a FV activity assay to complement immunoblotting techniques in order to demonstrate that inactivation of FV is associated with proteolytic cleavage by an *E. coli* protease.

ii) Purify, identify, and characterize the protease from the culture supernatant of *E. coli* O86a:K61 which is capable of cleaving and inactivating human FV in plasma.

iii) Assess the clinical relevance of FV inactivation in patients suffering from sepsis-induced DIC.

iv) Assess the prevalence of *E. coli* strains which are capable of inactivating FV.

v) Elucidate the role(s) of proteases as part of a common virulence mechanism used by pathogenic *E. coli* to inactivate human coagulation FV and the host coagulation process.

Materials and Methods

Protein Quantification

Protein concentration was measured by absorbance at 280nm, the Bradford method, or the bicinchoninic acid (BCA) protein assay.

Absorbance at 280nm

Samples of interest were added to a 96-well UV compatible plate (Costar, Mississauga, ON), to a final volume of 200µl with or without dilution. The samples were blanked against 200µl of the same buffer. Absorbance was measured at 280nm in a microplate-reader (Molecular Devices, Sunnyvale, CA) and the sample absorbance measurement was corrected with buffer blanks. Protein concentration was determined using a path length of 0.58cm and an extinction coefficient of 1mg/ml/cm with the Beer–Lambert law equation. All samples were diluted in buffer to give an absorption measurement less than 1.0 unit.

Bradford Method

The Bradford assay was performed according to Bradford (1976). In brief, 20µl of sample was added to 180µl of Bradford dye (0.5 mg/ml Coomassie Brilliant Blue G-250, 25% methanol, and 42.5% H_3PO_4) (Bio-Rad, Mississauga, ON) in a 96-well plate (Costar, Mississauga, ON) and incubated for 30 minutes at room temperature. The absorbance at 595nm was measured with a microplate-reader (Molecular Devices, Sunnyvale, CA). Samples were compared to a bovine serum albumin (BSA) standard (Bio-Rad, Mississauga, ON) of known protein concentration which was diluted in the same buffer as the samples.

BCA Assay

The BCA assay was performed according to the manufacturer's instructions (Thermo Scientific, Nepean, ON). In brief, 100µl of sample (Diluted when necessary) was added to 100µl BCA dye, in a 96-well plate (Costar, Mississauga, ON), and incubated at 37°C for 2 hours. The absorbance at 562nm was measured and corrected versus a solution containing only buffer. Samples were compared to a BSA standard (Fisher, Nepean, ON) diluted in the same buffer as the samples.

Activity Assays

Prothrombin Times (PT)

For this assay, 50µl normal human pooled reference plasma (NHP), 50µl HBS (20mM HEPES, 150mM NaCl, pH 7.4), and 50µl thromboplastin (Trinity Biotech, Wicklow, Ireland) were added to removable strips (Nunc, Roskilde, Denmark) in a 96-well format. The samples were then incubated at 25°C for 1 minute with shaking between 15-25 seconds of the 1 minute incubation. Calcium chloride (50µl of 25mM) was then added to the sample(s). After a 15 second incubation at 25°C, the samples were agitated for 5 seconds, and the absorbance at 405nm was read every 5 seconds for 6 minutes in a microplate-reader (Molecular Devices, Sunnyvale, CA). PTs were determined as the time to reach the inflection point of the sigmoidal absorbance versus time curve produced.

Activated Partial Thromboplastin Time (aPTT)

For this assay, 50µl NHP, 50µl HBS, and 50µl aPTT reagent (bioMerieux, St Laurent, QC) were added to removable strips (Nunc, Roskilde, Denmark) in a 96-well format. The samples were then incubated at 25°C for 5 minutes, with 15 seconds of agitation during the 5 minute incubation, and 50µl of 25mM $CaCl_2$ was then added to the samples. After a 15 second incubation at 25°C, the samples were agitated for 5 seconds, and the absorbance at 405nm was read every 5 seconds for 6 minutes in a

microplate-reader (Molecular Devices, Sunnyvale, CA). The aPPTs were determined as the time to reach the inflection point of the sigmoidal absorbance versus time curve produced.

E. coli Protease Activity in the aPTT Assay

For this assay, 60µl of concentrated *E. coli* culture supernatant was incubated with 60µl of NHP (Precision Biologicals, Halifax, NS) for 30 minutes at room temperature, and 100µl of the 120µl reaction was added to 50µl aPTT reagent (bioMerieux, St Laurent, QC) and assayed in aPPT assay described above.

E. coli Protease Activity Assay in Whole Blood

For this assay, whole human blood (9 vol) was collected from a consenting volunteer (Male, 25yrs) into 3.2% tri-sodium citrate (1vol) with a 20 gauge needle using a syringe (Becton Dickenson, Franklin Lakes, NJ). Concentrated *E. coli* supernatant (60µl) was incubated with 60µl of whole human blood for 30 minutes at room temperature. A portion of the 120µl (100µl) reaction was added to 50µl thromboplastin (Trinity Biotech, Wicklow, Ireland), and 50µl 25mM $CaCl_2$ reagent with a ball bearing in a 4ml culture tube (Starstedt, Montreal, QC). The sample was agitated and repeatedly immersed in a 37°C water bath. Clot times were determined as the time after $CaCl_2$ addition to retard ball bearing motility within the tube. All assays were performed within 1 hour of drawing the blood sample and citrated fresh blood was stored at room temperature until use.

1-Stage FV Activity Assay in Human Plasma

The FV microplate activity assay in human plasma has been described elsewhere (Tilley et al 2011).

FV deficient plasma was generated from normal human plasma according to (Blood et al 1979). Briefly, whole blood (9vol) was drawn from a consenting volunteer (Male, 50yrs) into tri-sodium citrate

(1vol) with a 20 gauge needle (Becton Dickenson, Franklin Lakes, NJ), and centrifuged twice at 3300 x g for ten minutes at room temperature. Cell-free plasma was collected, and EDTA was added to 5mM with gentle stirring at room temperature. After correcting the pH to 7.4, the plasma was then incubated at 37°C for approximately 14 hours. The prolongation of the prothrombin time was monitored every hour until it reached a stable maximal value.

For this assay, 50µl of FV deficient plasma was added to 96-well microplate removable strips (Nunc, Roskilde, Denmark), followed by an addition of 50µl of 40-fold diluted NHP or sample plasma in HBS, and an addition of 50µl extensively dialyzed (1:100 20mM Tris, 150mM NaCl, pH 7.5) thromboplastin (Trinity Biotech, Wicklow, Ireland). The samples were then incubated at 25°C in a microplate-reader (Molecular Devices, Sunnyvale, CA) for 1 minute, with agitation for 10 seconds after the first 15 seconds of incubation. Calcium chloride (50µl of 25mM) was then added, and the samples were incubated for an additional 15 seconds at 25°C, followed by 5 seconds of agitation. The absorbance at 405nm was read every 5 seconds for 6 minutes in a microplate-reader (Molecular Devices, Sunnyvale, CA). Clot times were determined as the time after $CaCl_2$ addition to reach the inflection point of the sigmoidal absorbance versus time curve comprising the clot formation event. A Log-Log standard curve of clot time versus FV activity was generated using serial dilutions of NHP in HBS. 1U of FV activity was defined as the FV activity present in 1ml NHP. The initial rate of clot formation was determined using linear regression of the first 5 points after clotting was initiated. A Log-Log standard curve of initial rate versus FV activity was generated using serial dilutions of NHP in HBS. The extent of clot formation was determined as the maximal absorbance reading minus the minimal absorbance reading during the clot formation event. A standard curve of extent of clot formation versus FV activity was generated using serial dilutions of NHP in HBS.

2-Stage FV Activity Assay in Human Plasma

For this assay, NHP or sample plasma was diluted 100-fold in HBS containing 2.83mM $CaCl_2$ (Final concentration). Human thrombin was prepared according to Bajzar et al (1995) and was diluted in HBS and added to samples to give a final concentration of 0.5U/ml (Approximately 10nM). After thrombin addition, the samples were incubated for 1 minute at 37°C. The samples were briefly vortexed, and 50µl was assayed in the 1-stage FV Activity Assay in Human Plasma described above.

E. coli Protease Activity versus FV in Human Plasma

For this assay, 30µl of *E. coli* culture supernatant was incubated with 30µl of NHP diluted 5-fold in HBS, for 30 minutes at room temperature. The mixture was diluted an additional 4-fold in HBS (40-fold final dilution with respect to NHP) and 50µl of the 40-fold diluted sample was assayed using 1-stage FV Activity Assay in Human Plasma described above.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

For this assay, 20-25µl of sample was added to 15-20µl 2x loading dye (LD; 0.025% bromophenol blue, 12.5% glycerol, 2% SDS, 5% β-mercaptoethanol, 10mM HEPES, 75mM NaCl) to a final volume of 40µl. The samples were incubated at 95°C for 5 minutes, and centrifuged at 4,000 x g for 4 seconds. The samples were loaded into polyacrylamide 4-20% gradient gels (Bio-Rad, Mississauga, ON) with 5µl-20µl pre-stained molecular weight standards (Bio-Rad, Mississauga, ON) added to the two outer wells. The gels were electrophoresed at 150V constant voltage for approximately 1.5h, in 25mM Tris, 192mM glycine, 0.1% SDS, pH 8.3. The gels were then stained with either Coomassie Brilliant Blue (CBB) or silver.

Coomassie Brilliant Blue Staining for SDS-PAGE

The gels were incubated in dilute CBB stain (0.01% CBB, 13% methanol, 5% acetic acid) for approximately 16 hours, at room temperature. The gels were de-stained for at least 1 hour in de-stain solution (19% methanol, 10% acetic acid) and photographed.

Silver Staining for SDS-PAGE

This procedure was modified from Merril et al (1981). Briefly, 200ml prefix solution (50% methanol, 10% ethanol, in water) was added to the gel and microwaved (Danby designer model) for 1.5min at 50% power, the gel was removed during the 1.5 minute microwave step and shaken frequently at room temperature for approximately 10 seconds every 30 seconds. The gel was then incubated in the prefix solution at room temperature for 2 minutes with agitation. The prefix solution was decanted, replaced with 200ml distilled water, and microwaved at 50% power with frequent shaking. The gel was then incubated in distilled water at room temperature for 2 minutes with agitation. The water was decanted and replaced with a solution of 100µM dithiothreitol (DTT solution) and then microwaved for 2 minutes at 50% power with frequent shaking. The gel was then incubated in the DTT solution at room temperature for 2 minutes with agitation. The DTT solution was decanted and replaced with silver nitrate solution (0.1% $AgNO_3$ (w/v) in water) and microwaved for 1.5 minutes at 50% power with frequent shaking. The silver nitrate solution was decanted, and the gel was washed twice with 200ml of distilled water. Developer solution (200ml of 3% sodium carbonate, 0.019% formaldehyde) was added and incubated at room temperature with agitation until the protein bands reached a desirable intensity. The developer solution was then decanted and replaced with 10ml 2.3M citric acid and agitated for 1 minute to stop the reaction. The citric acid solution was decanted, and the gel was washed with 200ml of distilled water. The water was decanted and the gel was stored in 0.03% sodium carbonate for at least 1 hour prior to being photographed.

Western Blotting

For this assay, 30µl of sample or HBS was incubated with 30µl NHP diluted 5-fold in HBS for 30 minutes at room temperature. HBS (420µl) was then added, and the diluted samples (100µl) were added to 100µl of the 2x LD. The samples were then incubated at 95°C for 5 minutes, and centrifuged at 4,000 x g for 4 seconds. Samples (40µl) containing 0.25µl plasma were loaded into the wells of a 4-20% polyacrylamide gradient gel (Bio-Rad, Mississauga, ON). Pre-stained molecular weight standard (20µl) (Bio-Rad, Mississauga, ON) were added to each of the outer two wells. The gel was electrophoresed at 150V, constant voltage for approximately 1.5 hours in 25mM Tris, 192mM glycine, 0.1% SDS, pH 8.3. The gels were removed and electroblotted onto polyvinylidene fluoride (PVDF) Immobilon-P membranes (Millipore, Etobicoke, ON) at 35V constant voltage at 4°C with gentle stirring for approximately 16 hours in 25mM Tris, 192mM glycine, 0.05% SDS, 10% methanol, pH 8.3. The PVDF membrane was then incubated in blocking buffer, 0.1% Tween-20 in Phosphate Buffer Saline (14mM NaCl, 1.5mM KH2PO4, 10mM NaHPO4, 2.5mM KCl, pH 7.4) (PBS_{TW}) containing 2% BSA (Fisher, Nepean, ON) at room temperature for 1 hour with agitation. The blocking buffer was then decanted and the PVDF membrane was washed 4x 5 minutes in 200ml washing buffer (PBS_{TW}). The final wash solution was decanted and replaced with 150ml primary antibody solution (198ng/ml (37,500-fold diluted) sheep anti-human Factor V IgG (Haematech, Burlington, VT), 5% (w/v) no-fat milk powder, in PBS_{TW}) and incubated for 1 hour at room temperature with agitation. The primary antibody solution was decanted and the PVDF membrane was washed 4x 5 minutes in 150ml PBS_{TW}, at room temperature. The final wash solution was decanted and replaced with 200ml secondary antibody solution (30ng/ml (25,000-fold diluted) Donkey anti-Sheep IgG conjugated with horseradish peroxidase (Jackson Immunochemicals, West Grove, PA) in 5% no fat milk powder in PBS_{TW}). The secondary antibody solution was decanted and the PVDF membrane was washed 4x 5 minutes in 150ml PBS_{TW} followed by a 150ml distilled water wash for 5 minutes at room temperature. The water was decanted and replaced with 10ml each of enhanced

chemiluminescence (ECL) reagent 1 and 2 (PerkinElmer, Waltham, MA) for 1 minute at room temperature with gentle stirring. The PVDF membrane was then removed from the ECL solution, wrapped in plastic, and exposed to X-Ray film (Kodak, Toronto, ON) for various times at room temperature with an intensifying screen. The film was then developed with an automated developer (Kodak X-OMAT 1000A, Toronto, ON) and the image was scanned (Hewlett-Packard Scanjet 4750c scanner, Mississauga, ON).

Column Chromatography

Q-Sepharose Anion Exchange Chromatography

Q-Sepharose (GE Healthcare, Baie d'Urfe, QC) (1-2ml) was prepared according to manufacturer's instructions. In brief, ethanol storage solution was decanted and replaced with running buffer (20mM HEPES, pH= 7.4 unless stated otherwise). The gel matrix was agitated into a slurry, loaded into a column, and allowed to settle to a total bed volume of approximately 1-2ml. The matrix was equilibrated in at least 50 column volumes running buffer, "charged" in at least 25 column volumes 1M NaCl in running buffer, and re-equilibrated in at least 50 column volumes running buffer. Sample was then applied to the column and incubated for 0.5h at room temperature, or for 16h at 4°C. The column was washed with 10ml running buffer, and the sample was eluted in a continuous salt gradient from 0-0.5M NaCl in running buffer (40ml total) at a flow rate of approximately 1.5ml/min. The columns were then washed with 10ml 1M NaCl and/or 70% ethanol in running buffer to remove tightly bound material. The column was stored in 0.01% sodium azide at 4°C between uses.

Sephadex G-100 Gel Filtration Column Chromatography

Approximately 40ml of Sephadex G-100 matrix (Pharmacia, Uppsala, Sweden) was washed and slurried into a 1x40 cm column according to manufacturer's instructions. The column was washed in at

least 100ml elution buffer (20mM Hepes, 150mM NaCl, 0.5M urea, 0.01% TritonX-100, 0.01% β-mercaptoethanol) prior to applying the sample. The sample (600µl) was applied (1:67 ratio sample to column volume) to the column, and 500µl fractions were collected at a flow rate of 0.25ml/min at room temperature.

Standard *E. coli* Culture Conditions

Unless stated otherwise, *E. coli* O86a:K61 (ATCC 33985) was grown in 10ml minimal A (MA) minimal media (60mM K2HPO4, 33mM KH2PO4, 7.5mM ammonium sulphate, 1.7mM trisodium citrate, 1mM MgSO4, 0.2% (w/v) glucose, pH 7.0), supplemented with 0.006% tryptone (Miller, 1992), overnight at 37°C, and sub-cultured (1% by volume) into 1L MA minimal media supplemented with 0.006% tryptone. The 1L cultures were grown for approximately 16 hours at 37°C without agitation to an optical density (OD) at 600nm of approximately 0.6 ± 0.05 units.

Standard *E. coli* Protease Purification

E. coli O86a: K61 1L cultures were adjusted to pH 7.0, centrifuged (10,000xg, 30 minutes at 4°C) and 0.22µm filtered (Starstedt, Montreal, QC) to remove any remaining cells. The cell-free supernatant was concentrated from 1L to approximately 15ml using tangential flow filtration (TFF) (Pall, Mississauga, ON) with a 100kDa molecular weight cut off (MWCO) filter. If required, the concentrated supernatant was buffer exchanged directly in the TFF system by the addition of 1.5L of desired buffer, and re-concentration to 15ml. After removal of the 15ml concentrate, the system was washed by the addition of another 15ml of desired buffer. The solution was combined with the original 15ml concentrate, and concentrated further by centrifugal filter flow at 3,200 x g for 10 minutes at room temperature (CFF) with a 100kDa MWCO filter (Millipore, Etobicoke, ON) to 2-10ml.

E. coli Clinical Isolate Screen

E. coli JM109, *E. coli* O86a: K61, and 6 *E. coli* clinical isolates (3 from rectum, 2 from urine, and 1 from a wound), (Kindly provided by Helene Goulding, Medical Laboratory Science program, UOIT), were inoculated into 1mL Luria-Bertani broth (LB) overnight at 37°C 225rpm, streaked onto LB agar plates, and grown overnight at 37°C to confirm single colony morphology type. LB (50ml) was inoculated with a single bacterial colony from the plates and grown for 16h at 37°C 225rpm. Cells were removed by centrifugation at 10,000xg for 30 minutes at 4°C, and 0.22µm filtration (Millipore, Etobicoke, ON). The cell-free supernatant was concentrated from 50ml to 1ml using CFF on 100kDa MWCO filter (Millipore, Etobicoke, ON).

Transmission Electron Microscopy (TEM)

A 1L culture of *E. coli* O86a:K61 was grown in MA minimal media to an optical density of approximately 0.6 at 600nm. Cells were removed by centrifugation at 10,000xg for 30 minutes at 4°C and 0.22µm filtered (Starstedt, St. Leonard, QC). The cell-free supernatant was concentrated from 1L to 30ml using TFF with a 100kDa MWCO filter (Pall, Mississauga, ON), and further concentrated using CFF on 100kDa MWCO filters (Millipore, Etobicoke, ON) to 5ml. The concentrated material was ultracentrifuged at 150,000xg for 1.5h at 4°C and the pellet was suspended in 250µl HBS. The re-dissolved pellet was analyzed directly or after 100-fold dilution in HBS. The sample was applied to an ionized carbon-coated formvar film which was attached to a metal specimen grid for 30 seconds, and then washed with water for 30 seconds. The sample was stained with 2% uranyl acetate, and washed again with water for 30 seconds prior to imaging. TEM imaging was performed at the Pathology & Laboratory Medicine department of the Hospital for Sick Children (Toronto, ON) by Doug Holmyard.

N-Terminal Sequencing

Samples were prepared according to Standard *E. coli* Protease Purification protocol (See above). The concentrated *E. coli* culture supernatant (5ml) was ultracentrifuged at 150,000xg for 1.5 hours at 4°C. The ultracentrifuged concentrated *E. coli* culture supernatant was then concentrated to 1ml using CFF with a 100kDa MWCO filter (Millipore, Etobicoke, ON) and subjected to Sephadex G100 gel filtration column chromatography as described above. Fractions with the highest specific activity versus FV in NHP were pooled and concentrated 8-fold (800µl to 100µl) using CFF on a 10kDa MWCO filter (Millipore, Etobicoke, ON). The samples were added to an equal volume of 2x LD, and electrophoresed on 4-20% gradient polyacrylamide gels (Bio-Rad, Mississauga, ON) as described above in the SDS-PAGE section. The samples were then blotted onto PVDF membrane (Millipore, Etobicoke, ON) as described above in the Western blotting protocol; and stained with the Coomassie Brilliant Blue protocol for SDS-PAGE gels described above. The PVDF membrane was wrapped in plastic, thermo-sealed, and sent to the Advanced Protein Technology Centre at the Hospital for Sick Children (Toronto, ON) for N-terminal sequencing using the Edman degradation method (Edman, 1950).

Statistical Analysis

All statistical analysis was performed using Sigma Plot 11.0.

Results

A typical depiction of the clot formation event in the FV coagulation assay over time generated by the microplate reader is shown in Figure 3. Visual inspection of the wells after the assay was performed confirmed that clot formation took place. All reactions produced a sigmoidal curve with a typical change in absorbance of 0.35-0.45 absorbance units at 405nm between the starting absorbance and maximal absorbance caused by the formation of fibrin. At least three parameters may be compared between samples. (1) The clot time was defined as the time to reach half the maximal change in absorbance (Approximately the inflection point of the absorbance versus time sigmoidal curve). (2) The initial rate was defined using linear regression of the first 5 time points after clotting is initiated in the linear portion of absorbance versus time sigmoidal curve. (3) The extent of clotting was defined as the maximal absorbance minus the initial absorbance of the sigmoidal absorbance versus time curve.

To determine the FV clotting activity in a plasma sample, a standard curve of the time for clot formation versus FV 1-stage activity was generated using 2-fold serial dilutions of NHP into HBS. Fitting the Log-Log plot of clot time versus FV 1-stage activity demonstrated a strong linear relationship between these variables after regression analysis (r^2 = 0.980) (Figure 4, Panel A). The relationship of clot time versus FV 1-stage activity remained linear in NHP diluted up to approximately 1024-fold. Fitting the Log-Log plot of initial rate versus FV 1-stage activity also demonstrated a strong linear relationship between these variables after regression analysis (r^2 = 0.980) (Figure 4, Panel B). Given that the FV concentration in NHP is approximately 12-40nM (Tracy et al 1982), the results indicate that the assay is sensitive to approximately 24-80pM FV in NHP. The results indicate that the normal range of FV activity in the FV 1-stage activity assay in 15 healthy controls (Male and female, aged 18-25) was approximately (Mean± Standard Deviation; Range): 0.96±0.14 U/ml; 0.68-1.11 U/ml. This result is similar to the FV

Figure 3 Monitoring Clot Formation in NHP with Kinetic Microplate FV 1-stage Coagulation Assay.

Fibrin clot formation in NHP was monitored at 405nm over time in a microplate reader. The profile represents a typical microplate reader output of a 6 minute reaction of 32-fold diluted NHP. Readings were taken every 5 seconds. The y-axis denotes the change in absorbance at 405nm that occurred as a result of clot formation in plasma. The time of fibrin formation was defined as the time to reach the half maximal increase in absorbance (The inflection point; 36.4 seconds). The Initial rate of clot formation was defined as the rate of change of absorbance at 405nm over the first 5 time points of linear increase of absorbance (611.88 mUnits/min). The extent of clot formation was defined as the difference between the maximum and minimum absorbance at 405nm (0.35 Units).

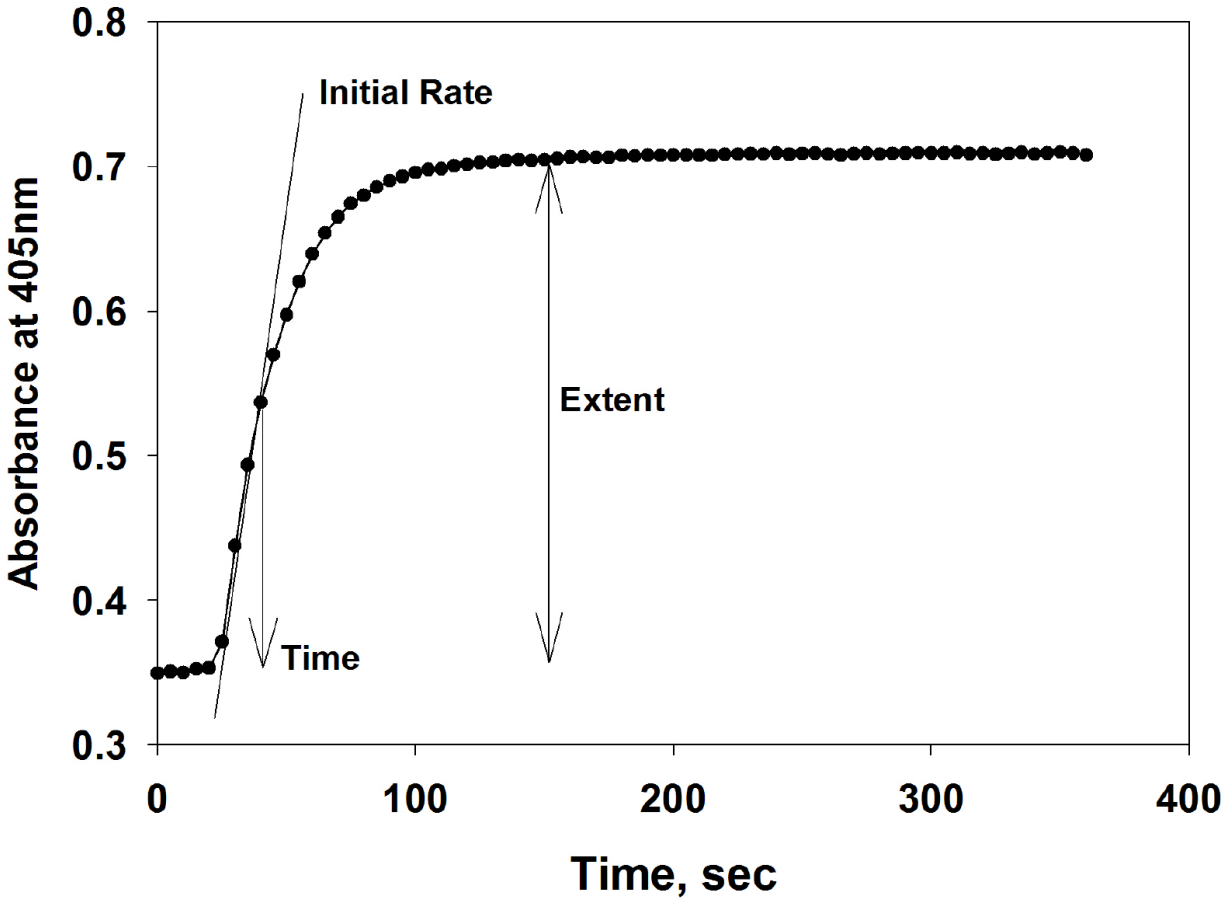
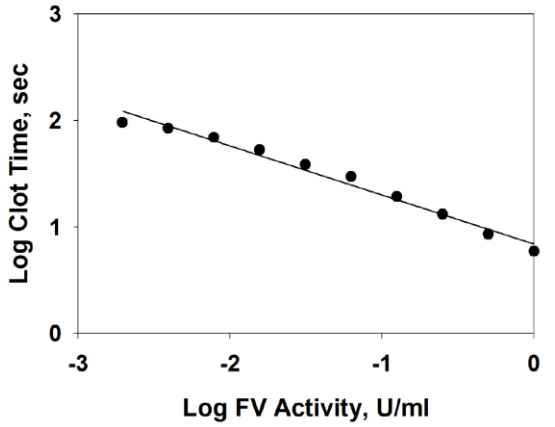

0.8
0.7
0.6
0.5
0.4
0.3
Absorbance at 405nm
Initial Rate
Extent
Time
0
100
200
300
400
Time, sec

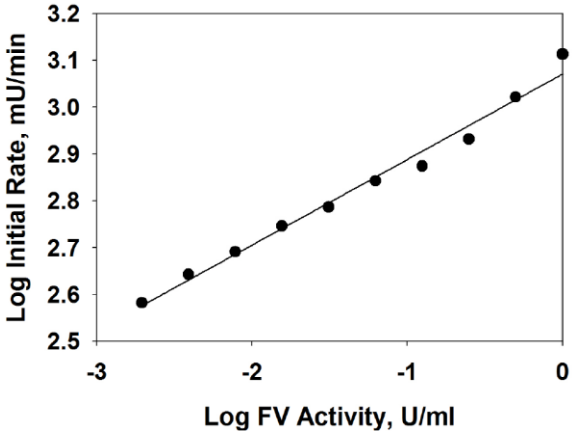
Figure 3.

Figure 4 Standard Curve of Time of Clot Formation versus Factor V Activity in NHP using FV 1-stage Microplate Assay. NHP was serially diluted (0- to 1024-fold in HBS) and assayed with the FV 1-stage microplate assay as described in text. Panel A denotes a Log-Log plot of the time of clot formation versus FV activity. Linear regression modelling of these results indicated a strong relationship between these variables (r^2= 0.980). Panel B denotes a Log-Log plot of the initial rate of clot formation versus FV activity. Linear regression modelling of these results indicated a strong relationship between these variables (r^2= 0.983). Panel C denotes a plot of extent of clot formation versus FV activity.

A)


Log Clot Time, sec
3
2
1
0
-3
-2
-1
0
Log FV Activity, U/ml

B)


Log Initial Rate, mU/min
3.2
3.1
3.0
2.9
2.8
2.7
2.6
2.5
-3
-2
-1
0
Log FV Activity, U/ml

C)

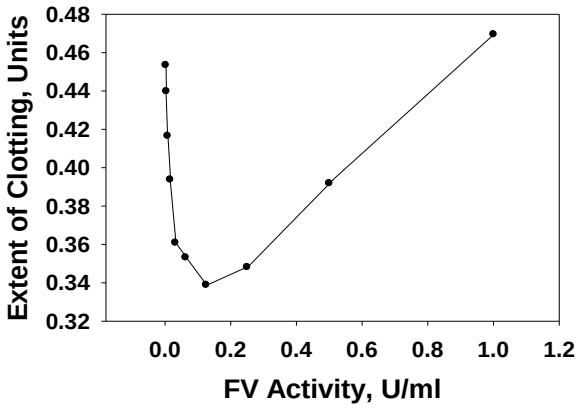

Extent of Clotting, Units
0.48
0.46
0.44
0.42
0.40
0.38
0.36
0.34
0.32
0.0
0.2
0.4
0.6
0.8
1.0
1.2
FV Activity, U/ml

Figure 4.

activity and range (0.66-1.14 U/ml) in healthy controls reported with automated analyzers (Cutler et al 2010). A standard curve of the extent of clot formation versus FV 1-stage activity is also reported (Figure 4, Panel C); however, given the parabolic nature of the curve, it was not suitable to calculate FV activity in a given plasma sample.

Under the conditions of the assay using 40-fold diluted NHP, the intra-assay variability of the time of clot formation in the FV 1-stage assay among 6 wells on 8 different days was 3.4%. The inter-assay variability of the time of clot formation in the FV 1-stage assay among 6 wells on 8 different days was 7.1%. Thus, the intra- and inter-assay variability of the FV 1-stage assay was at a low and acceptable level.

The standard curve of clot time versus FV 1-stage activity (Figure 4, Panel A) was used to measure the FV activity in nine DIC patient plasmas which were (FV 2-stage activity) or were not (FV 1-stage activity) intentionally activated with added thrombin. The extent and initial rate of clot formation was also analyzed (Table 1). All nine DIC patient plasmas exhibited FV 1-stage activities and initial rates that were decreased on average by 54% and 18%, respectively, from NHP. The extents of clot formation in the FV 1-stage assay in the DIC patients were not largely different from NHP, and increased on average by approximately 13% from NHP.

Activation of NHP with thrombin generated an approximate 8-fold increase in FV 2-stage activity above the FV 1-stage activity (Table 1). This indicates that the FV in NHP was mainly present in its inactive procofactor form which is consistent with results published by other investigators (Nesheim et al 1981). The FV 2-stage and total activity (2-stage FV activity - 1-stage FV activity) were also decreased in the DIC patients on average by approximately 44% and 42%, respectively, from NHP. The initial rates and extents of clot formation in the 2-stage assay in the DIC patients were not largely different from NHP, and varied on average by approximately 9% and 4%, respectively, from that observed with NHP.

Table 1 FV Functional Activity in NHP and in 9 Patients Who Developed DIC. The FV 1-stage, 2-stage, and total activity in NHP and 9 DIC patient plasma samples were determined from the FV 1-stage microplate assay standard curve of time of clot formation versus FV activity. Also presented are the extent of clot formation and the initial rate of clot formation in the FV 1-stage and FV 2-stage assays.

Sample	1-stage assay Activity (Units/ml)	1-stage assay Extent (Units)	1-stage assay Initial Rate (mUnits/min)	2-stage assay Activity (Units/ml)	2-stage assay Extent (Units)	2-stage assay Initial Rate (mUnits/min)	Total activity (Units/ml)
NHP	1.02	0.363	744.96	7.93	0.433	375.84	6.91
Patient 1	0.36	0.404	583.80	3.84	0.432	249.96	3.48
Patient 2	0.67	0.416	704.04	5.28	0.469	323.16	4.61
Patient 3	0.31	0.435	562.08	3.92	0.453	294.96	3.61
Patient 4	0.39	0.435	617.04	4.14	0.462	372.60	3.75
Patient 5	0.73	0.401	641.40	5.91	0.433	354.24	5.18
Patient 6	0.45	0.403	600.72	4.16	0.445	393.96	3.71
Patient 7	0.49	0.395	575.40	4.89	0.449	357.48	4.40
Patient 8	0.19	0.448	489.00	2.89	0.450	330.48	2.70
Patient 9	0.64	0.423	699.48	5.11	0.455	417.24	4.47

Table 1.

To analyze the prevalence of *E. coli* strains which secrete proteases capable of cleaving and inactivating FV, 50-fold concentrated (CFF with a 100kDa MWCO filter) cell-free supernatants from JM109, O86a:K61, and 6 *E. coli* clinical isolates from Lakeridge Healthcare (Oshawa, ON) were subjected to FV Western Blotting (Figure 5). Also, the 100kDa filtrate from *E. coli* O86a:K61 was concentrated using centrifugal filter flow with a 10kDa MWCO filter (O86a: K61 FT) and analyzed. Two of six *E. coli* clinical isolates (Wound, and 2 Rectum) secreted a protease which was retained on a 100kDa filter and was capable of cleaving FV, again to a 250kDa product. Curiously, the laboratory strain *E. coli* JM109 also demonstrated the ability to cleave and inactivate FV in NHP. *E. coli* O86a: K61 culture supernatant 100kDa retenate, but not filtrate, was capable of cleaving and inactivating FV. This result agrees with data obtained from the *E. coli* protease activity versus FV in plasma assay where 95% of the protease activity was retained by the 100kDa filter (Data not shown).

To measure the *E. coli* secreted protease activity, a standard curve of FV 1-stage clot time versus protease activity (Figure 6) was generated using 2-fold serial dilutions in HBS of 500-fold concentrated *E. coli* culture supernatant. A rectangular 3-parameter non-linear regression indicated a strong relationship between these variables between 8-fold and 512-fold dilution points ($r^2 = 0.993$). At least one protease standard curve was generated for every culture preparation from the TFF 100kDa retenate. 1 unit of protease activity was defined as the activity present in 1ml 500-fold concentrated *E. coli* supernatant.

The effect of 500-fold concentrated *E. coli* supernatant on FV activity in NHP was measured using the FV 1-stage activity assay (Figure 7). In the presence of 0.1% β-mercaptoethanol the time of clot formation was increased on average by 55.2 seconds, compared to NHP treated with buffer. FV activity was determined using a FV standard curve (Figure 4). In samples treated with concentrated *E. coli*

Figure 5 Effects of Concentrated Supernatants from *E. coli* Strains JM109, O86a: K61, and 6 Clinical Isolates on FV by Western Blot. LB overnight *E. coli* cultures (50ml) supernatants of strains JM109 (Laboratory strain), O86a:K61, and 6 *E. coli* clinical isolates were concentrated as described in the text, and exposed to NHP. NHP (0.25µl) was loaded per well in 4-20% polyacrylamide gradient gels, and electrophoresed for 1.5h at 150V, constant voltage. Proteins were electroblotted onto a PVDF membrane for 16 hours at 35V, constant voltage, and were probed with sheep anti-human Factor V IgG, and then donkey anti-sheep IgG conjugated with HRP. See materials and methods for complete description of Western Blotting protocol. "NHP" refers to plasma which was treated with buffer alone. 250 refers to the migration distance of the 250kDa molecular weight standard.

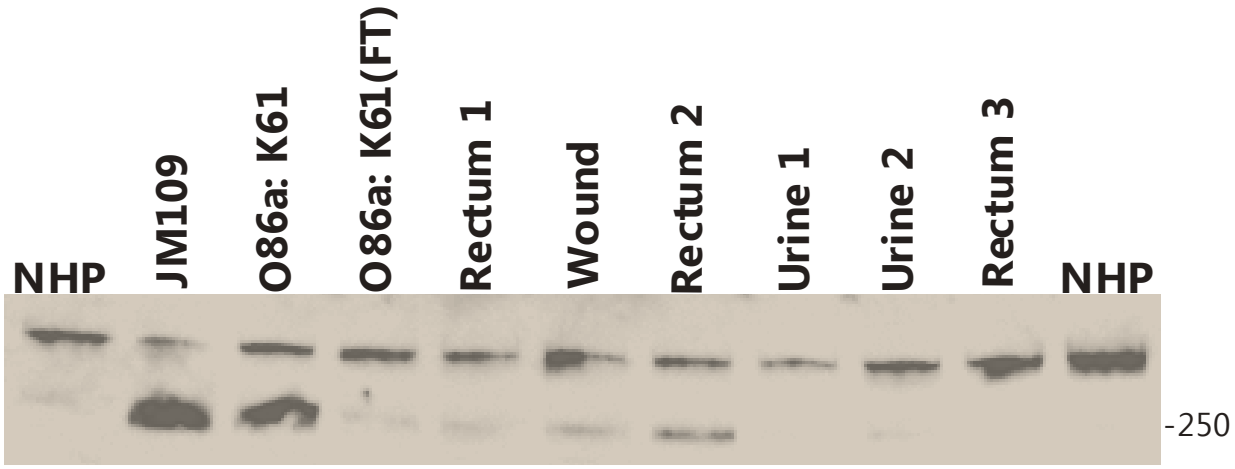

NHP
JM109
O86a: K61
O86a: K61(FT)
Rectum 1
Wound
Rectum 2
Urine 1
Urine 2
Rectum 3
NHP
-250

Figure 5.

Figure 6 Standard Curve of Time of Clot Formation versus *E. coli* Protease Activity in NHP using FV 1-stage Microplate Assay. *E. coli* concentrated culture supernatant was serially diluted (8- to 512-fold in HBS) and incubated with 5-fold diluted NHP in HBS for 30 minutes at room temperature. The sample was further diluted 4-fold (40-fold final with respect to NHP) and assayed with the FV 1-stage microplate assay described in text. The curve was generated using a rectangular 3-parameter non-linear regression model that indicated a strong relationship between these variables (r^2= 0.993).

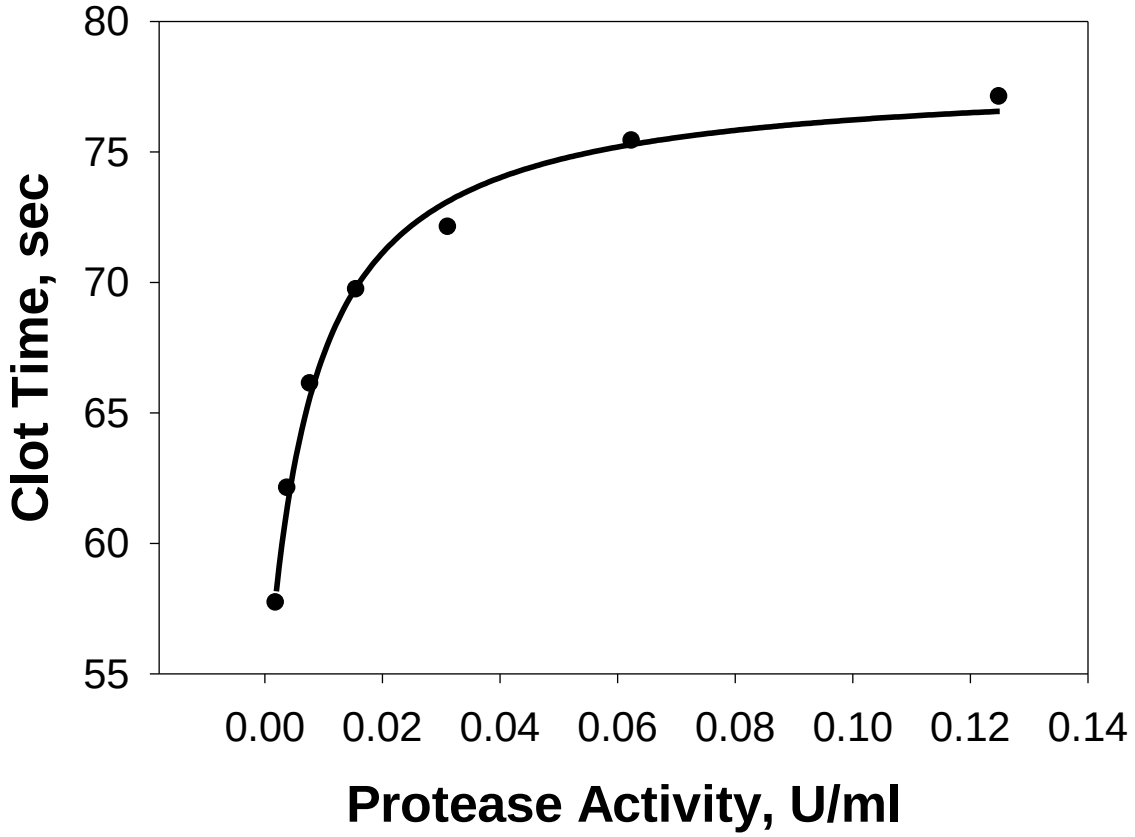

80
75
70
65
60
55
Clot Time, sec
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
Protease Activity, U/ml

Figure 6.

Figure 7 Effect of Concentrated *E. coli* Supernatant on FV Activity in NHP. Concentrated *E. coli* supernatant (500-fold) was prepared according to standard enzyme preparation described in text, and incubated with NHP according to *E. coli* protease activity assay versus FV described in text. FV activity was determined using FV 1-stage clot times and the FV standard curve (Fig. 4). FV activity was significantly reduced compared to samples treated with buffer alone (n=4, Paired Rank Sum Test, $p < 0.05$).

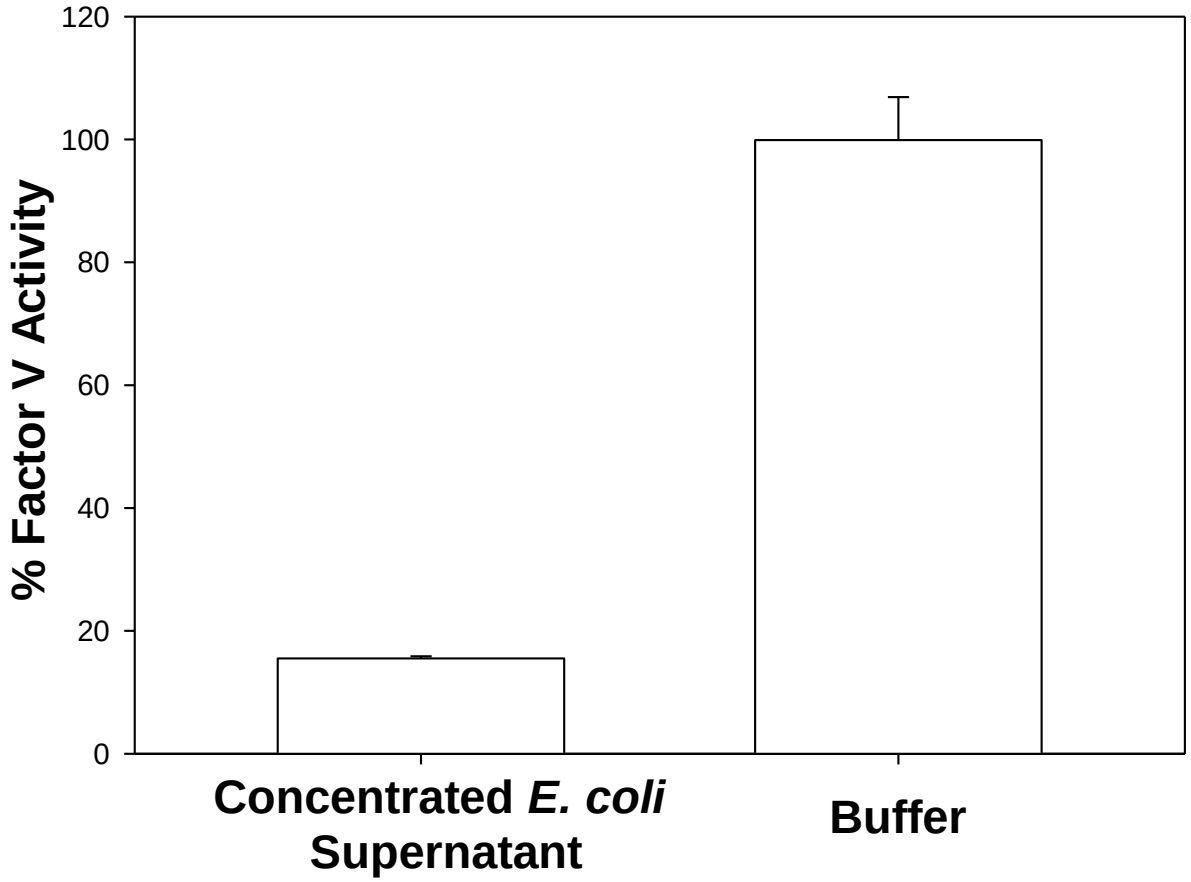

120
100
80
60
40
20
0
% Factor V Activity
Concentrated E. coli Supernatant
Buffer

Figure 7.

culture supernatant, FV activity was significantly decreased (Rank Sum Test, p = 0.026) to (Mean± Standard Deviation) 15% ± 0.34% (n=4), compared to the HBS buffer control. *E. coli* supernatant required concentration for accurate measurement of protease activity. Concentrated culture media without *E. coli* had no effect on FV activity in NHP, indicating that the inactivation effect required a component from growing bacterial cells which is released into the culture medium.

The effect of 500-fold concentrated *E. coli* supernatant on the initial rate of clot formation in NHP was measured using the FV 1-stage activity assay (Figure 8). The initial rate of clot formation was not significantly different (Paired t-Test, p = 1.0) in NHP treated with concentrated *E. coli* supernatant (Mean ± Standard Deviation) 334 mU/min ± 23mU/min, compared to NHP treated with buffer (Mean ± Standard Deviation) 334 mU/min ± 28mU/min (n=4).

The effect of 500-fold concentrated *E. coli* O86a:K61 supernatant on the extent of clot formation in NHP was measured using the FV 1-stage activity assay (Figure 9). The extent of clot formation was increased on average by 0.051 absorbance units (a405nm), compared to NHP treated with buffer. The extent of clot formation was significantly increased (t-test $p < 0.001$) to (Mean ± Standard Deviation) 111% ± 1% (n=4) compared to NHP treated with HBS.

The effect of 500-fold concentrated *E. coli* supernatant on the time of clot formation in whole human blood was measured using the *E. coli* protease activity versus whole blood assay (Figure 10). The time for clot formation was significantly increased (Paired t-Test, p = 0.047) when whole blood was treated with concentrated *E. coli* supernatant compared to samples treated with HBS. The time for clot formation was (Mean ± Standard Deviation) 41.3s ± 4.3s in samples treated with concentrated *E. coli* supernatant, compared to (Mean ± Standard Deviation) 35.8s ± 4.1s in buffer controls (n=6).

The effect of 500-fold concentrated *E. coli* supernatant on the time of clot formation was measured using the *E. coli* protease activity in aPTT assay (Figure 11). The time for clot formation was

Figure 8 Effect of Concentrated *E. coli* Supernatant on Initial Rate of Clot Formation in NHP.

Concentrated *E. coli* O86a:K61 supernatant (500-fold) was prepared according to standard enzyme preparation described in text and incubated with NHP according to *E. coli* protease activity assay versus FV described in the text. The initial rate of clot formation was determined as described in Figure 1. The initial rate of clot formation was not significantly different in samples treated with concentrated *E. coli* supernatant compared to samples treated with buffer alone (n=4, Paired t-Test, $p > 0.05$).

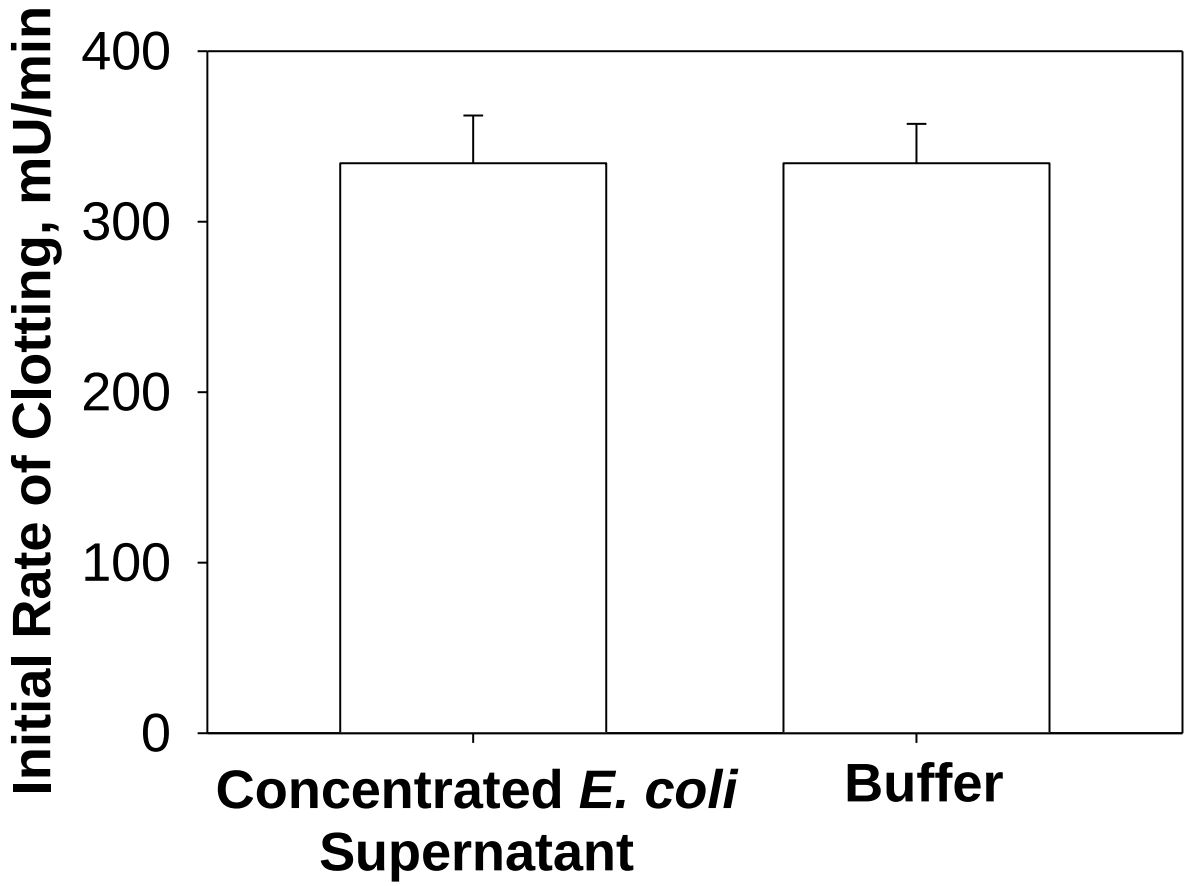

Initial Rate of Clotting, mU/min
400
300
200
100
0
Concentrated E. coli Supernatant
Buffer

Figure 8.

Figure 9 Effect of Concentrated *E. coli* Supernatant on Extent of Clot Formation in NHP. Concentrated *E. coli* O86a:K61 supernatant (500-fold) was prepared according to standard enzyme preparation described in text, and incubated with NHP according to *E. coli* protease activity assay described in text. The extent of clot formation was determined as described in Figure 3. The extent of clot formation was significantly increased in samples treated with concentrated *E. coli* supernatant compared to samples treated with buffer alone (n=4, Paired t-Test, $p < 0.05$).

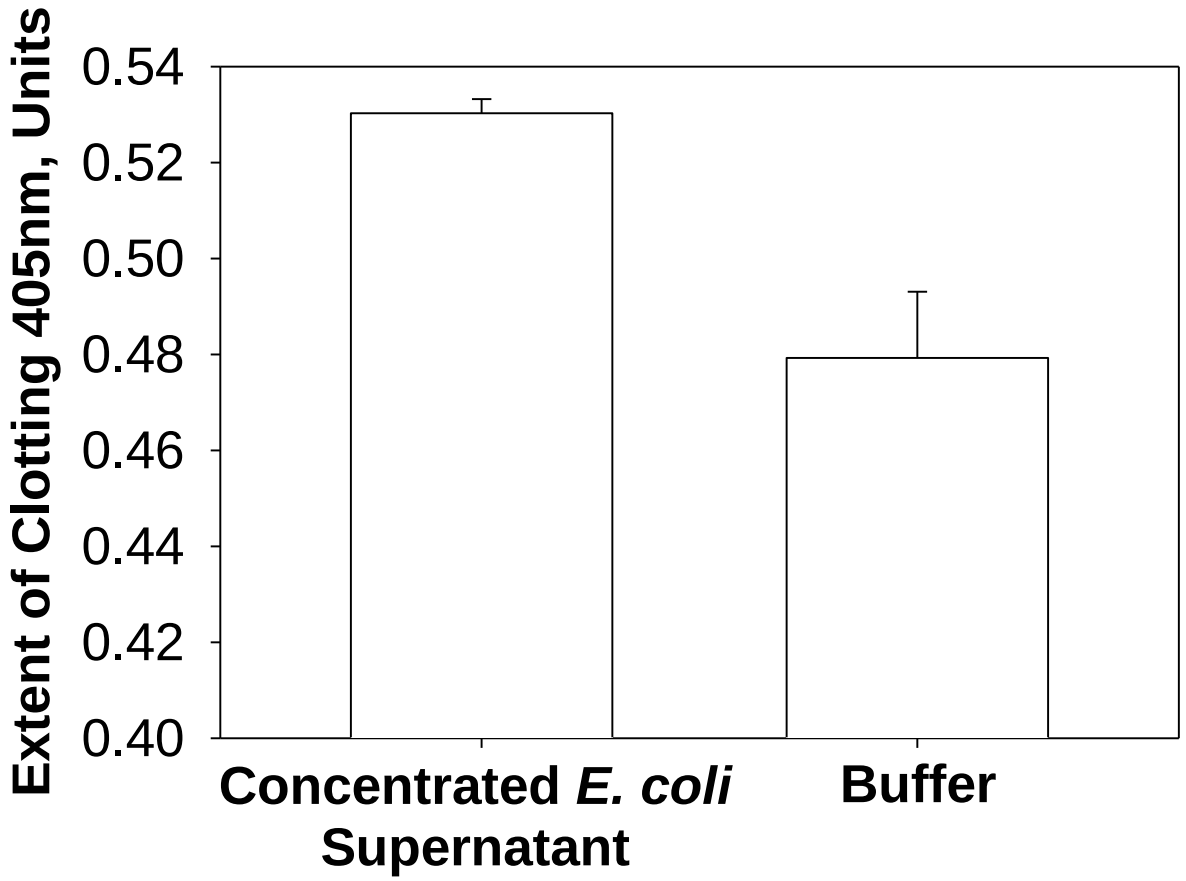

Extent of Clotting 405nm, Units
0.54
0.52
0.50
0.48
0.46
0.44
0.42
0.40
Concentrated E. coli Supernatant
Buffer

Figure 9.

Figure 10 Effect of Concentrated *E. coli* Supernatant on Time of Clot Formation in Whole Human Blood. Concentrated *E. coli* O86a:K61 supernatant (500-fold) was prepared according to standard enzyme preparation and incubated with whole human blood according to *E. coli* protease activity assay versus whole blood described in text. The time of clot formation was determined as the time to retard ball bearing motility within the reaction tube. Concentrated *E. coli* supernatant significantly increased the time for clot formation compared to samples treated with buffer alone (n=6, Paired t-Test, $p < 0.05$).

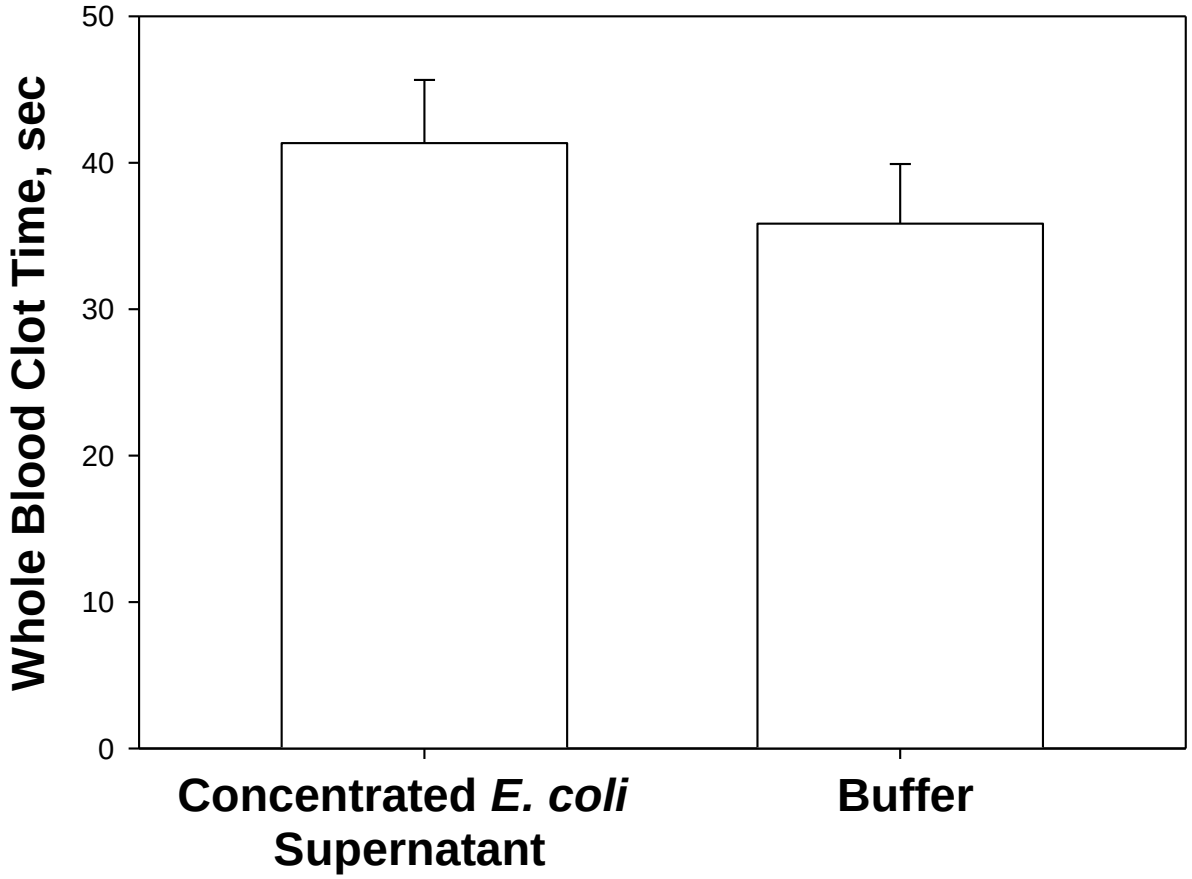

Whole Blood Clot Time, sec
50
40
30
20
10
0
Concentrated E. coli Supernatant
Buffer

Figure 10.

Figure 11 Effect of Concentrated *E. coli* Supernatant on Time of Clot Formation using aPTT Assay in NHP. Concentrated *E. coli* O86a:K61 supernatant (500-fold) was prepared according to standard enzyme preparation and incubated with NHP according to *E. coli* protease activity assay versus aPTT as described in text. The time of clot formation was determined as described in Figure 3. Concentrated *E. coli* supernatant significantly increased the time for clot formation compared to samples treated with buffer alone (n=3, Paired t-Test, $p < 0.05$).

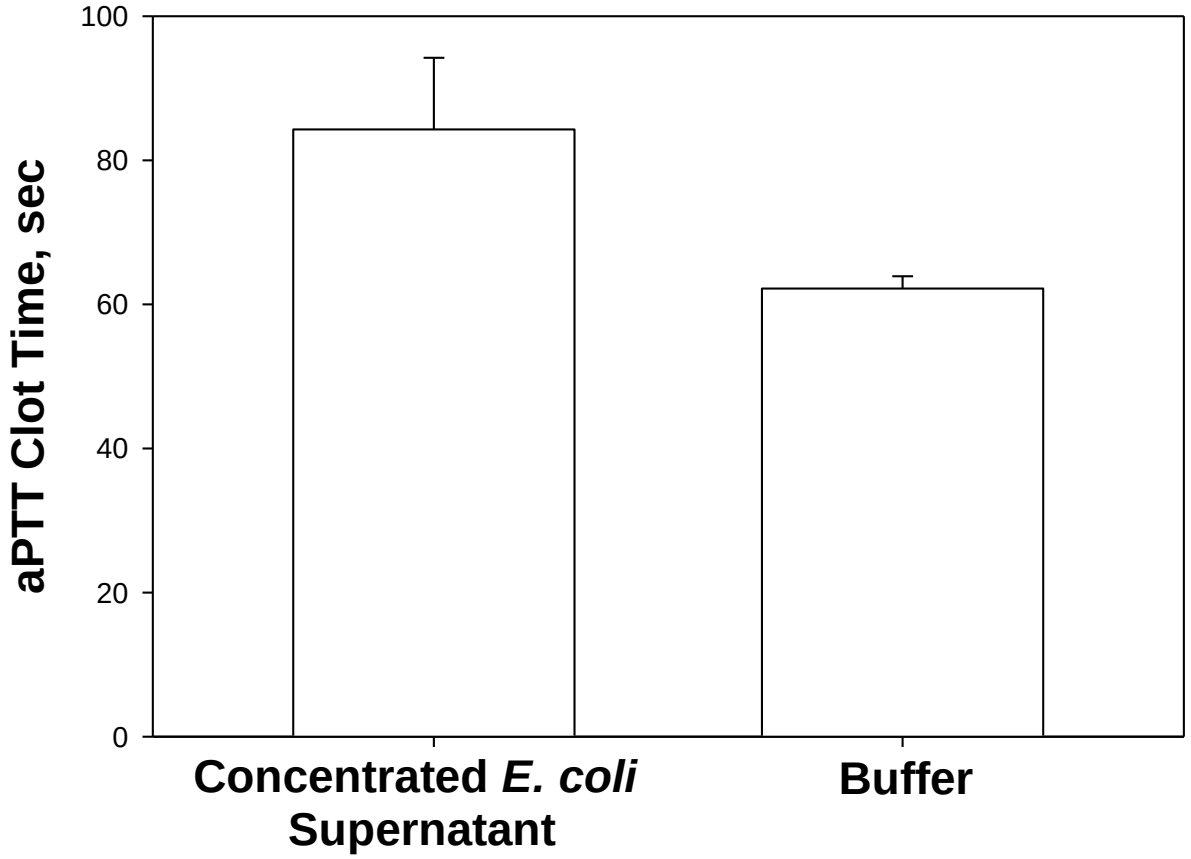

100
80
60
40
20
0
aPTT Clot Time, sec
Concentrated E. coli Supernatant
Buffer

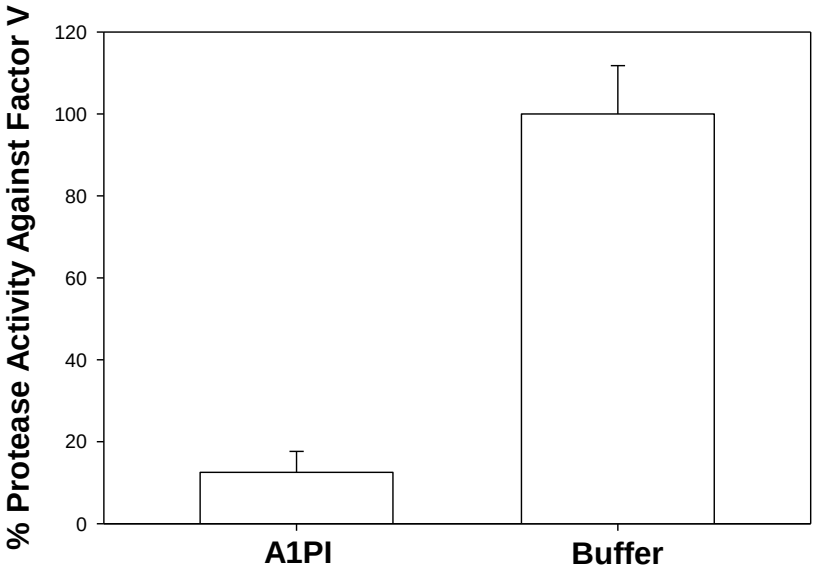
Figure 11.

significantly increased (Paired t-Test, p = 0.019) when NHP was treated with concentrated *E. coli* O86a:K61 supernatant, compared to samples treated with buffer alone. The time for clot formation was (Mean ± Standard Deviation) 84.3s ± 10s when NHP was treated with concentrated *E. coli* supernatant, compared to (Mean ± Standard Deviation) 62.2s ± 2s in buffer controls (n=3).

The effects of PMSF and A1PI on 500-fold concentrated *E. coli* supernatant were measured using the *E. coli* protease activity assay versus FV in NHP (Figure 12). *E. coli* protease activity was significantly reduced to (Mean ± Standard Deviation) 12.5% ± 5.1% of buffer control treated *E. coli* protease, after a 10 minute incubation with 1.5mg/ml A1PI at room temperature (n=3, Paired T-test $p<0.001$). A1PI at 1.5mg/ml had no significant effect on clot formation by itself, indicating that this inhibitory effect was directed at the *E. coli* protease; and not the coagulation factors during the microplate assay. *E. coli* protease activity was reduced to (Mean ± Standard Deviation) 75.7% ± 3.7% after a 1 hour incubation with 1.5mM PMSF at room temperature, compared to samples incubated with PMSF diluent alone. PMSF is an irreversible serine protease inhibitor that is extremely unstable in solutions of neutral pH, becoming completely inactive after 1 hour at room temperature. Therefore, a 1 hour incubation at room temperature was sufficient to prevent interference with the serine proteases of the coagulation system in the FV activity assay. The *E. coli* protease retained all of its activity after 30 minute incubations with 1mM tetracycline (Metallo protease inhibitor), 1mM N-[N-(L-3-transcarboxyirane-2-carbonyl)-L-Leucyl]-agmatine (E64) (Irreversible cysteine protease inhibitor), or 2mM iodoacetamide (Irreversible cysteine protease inhibitor) (Data not shown). The inhibitors alone had no effect on the FV activity assay indicating specificity for the *E.coli* protease. All inhibitor concentrations used were at or above the maximum suggested concentration recommended by the manufacturer. Tetracycline was used at the highest concentration tolerated by the activity assay which was comparable to the concentrations reported by others (Imamura et al 2001).

Figure 12 Effects of Protease Inhibitors on Concentrated *E. coli* Protease against FV Activity in NHP. Concentrated *E. coli* O86a:K61 supernatant (500-fold) was prepared according to standard enzyme preparation described in text, and incubated with equal volumes of 3 mg/ml alpha 1-protease inhibitor (A1PI; Calbiochem, LaJolla, CA) for 10 minutes (Panel A) or 3mM phenylmethylsulfonyl fluoride (PMSF) for 1 hour (Panel B). After incubation, samples were assayed using *E. coli* protease activity assay versus FV described in text. Activities were generated using the *E. coli* protease standard curve (Fig. 5) and compared to samples treated with buffer alone (Panel A: n=3, Paired t-Test, $p < 0.001$; Panel B: n=2).

A)


% Protease Activity Against Factor V
120
100
80
60
40
20
0
A1PI
Buffer

B)

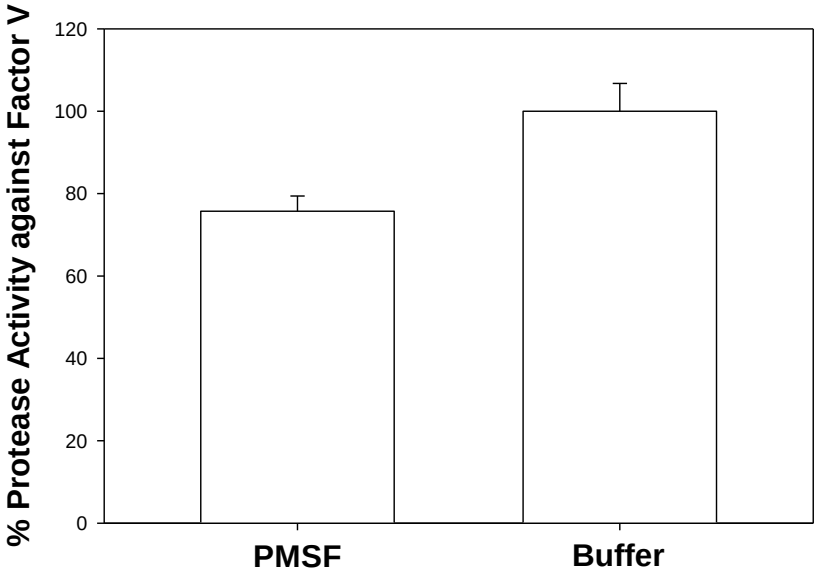

% Protease Activity against Factor V
120
100
80
60
40
20
0
PMSF
Buffer

Figure 12.

Figure 13 Effect of Divalent Cations on Concentrated *E. coli* Protease Activity in NHP. Concentrated *E. coli* O86a:K61 supernatant (500-fold) was prepared according to standard enzyme preparation described in text, and incubated with equal volumes 1mM $ZnS0_4$, $CuSO_4$, $FeCl_2$, or HBS for 10 minutes at room temperature. After incubation, samples were assayed using *E. coli* protease activity assay versus FV described in text. Activities were generated using the *E. coli* protease standard curve (Fig. 5) (n=3).

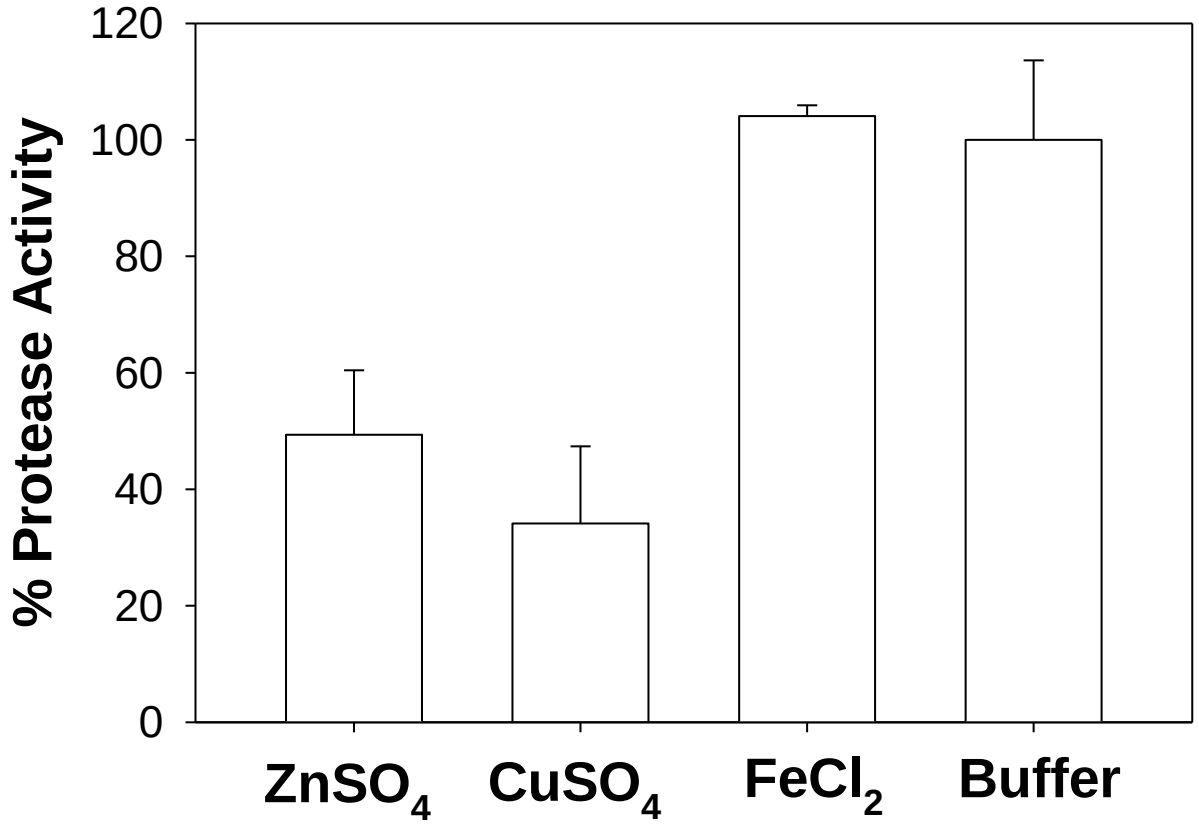

% Protease Activity
120
100
80
60
40
20
0
ZnSO4
CuSO4
FeCl2
Buffer

Figure 13.

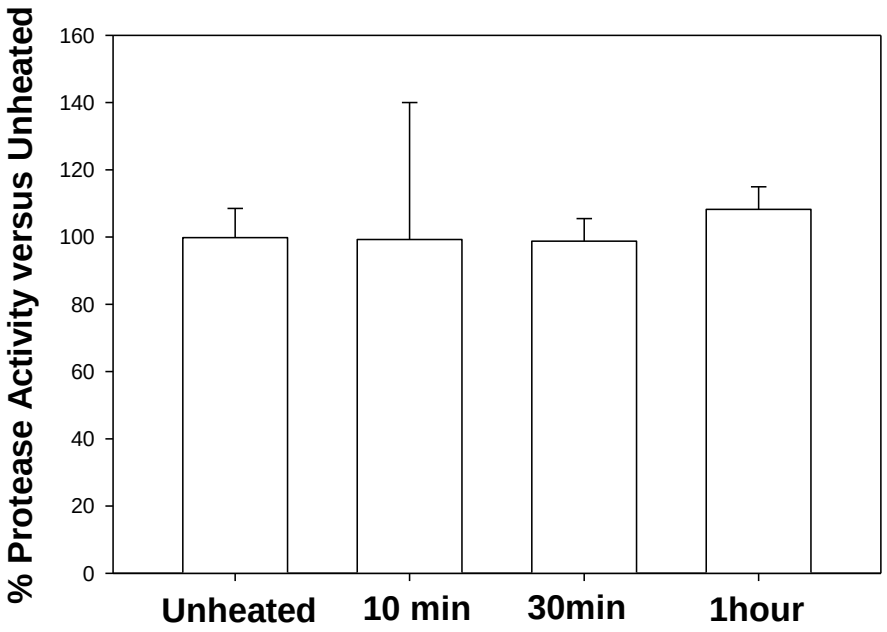
The effects of various divalent cations on 500-fold concentrated *E. coli* supernatant were measured using the *E. coli* protease activity assay versus FV (Figure 13) and compared to an *E. coli* protease standard curve (Figure 6). *E. coli* protease activity was (Mean ± Standard Deviation) 49% ± 11%, 34% ± 13%, or 104% ± 2% of untreated samples after 10 minute incubations at room temperature with 0.5mM $ZnS0_4$, $CuSO_4$, or $FeCl_2$, respectively, (n=3).

The heat stability of 500-fold concentrated *E. coli* supernatant was analyzed using the *E. coli* protease activity assay versus FV (Figure 14) and compared to an *E. coli* protease standard curve (Figure 6). The *E. coli* protease was stable at 80°C for up to 1 hour (Figure 14, Panel A), retaining (Mean ± Standard Deviation) 99% ± 40%, 99% ± 7%, and 108% ± 7% activity after 10 minutes, 30 minutes, and 1 hour at 80°C, respectively, compared to samples which were incubated at room temperature. The *E. coli* protease was somewhat susceptible to heat inactivation at 95°C (Figure 14, Panel B), retaining (Mean ± Standard Deviation) 87% ± 2%, 46% ± 3%, and 65% ± 8% activity after 10 minute, 1 hour, or 3 hour incubations, respectively, at 95°C. The *E. coli* protease was also stable for up to 1 hour at 70°C, and retained (Mean± Standard Deviation) 71%± 22% activity after 3 hour incubations at 70°C, compared to samples incubated at room temperature (Data not shown). Curiously, no protein visibly precipitated out of the solution after heating for up to 3 hours at up to 95°C (Data not shown).

A typical protease elution profile upon Sephadex G-100 gel filtration chromatography is shown in Figure 15. Approximately 82% of the *E. coli* protease activity applied to the column was recovered, and approximately 150% of the protein applied to the column was recovered. This was most likely due to protein concentration flanking the main protein peak being lower than the ideal detection limit of the a280nm assay. The *E. coli* protease specific activity was increased by approximately 3-fold (1.48mg/ml applied to column to 4.27mg/ml in fraction 18), which may be a conservative estimation due to the overestimation of protein concentration. Analysis of fraction 18 by SDS-PAGE with Coomassie Brilliant Blue staining revealed two proteins with apparent molecular weights of 37kDa and 90kDa (Figure 16).

Figure 14 Thermal Stability of Concentrated *E. coli* Protease Activity against FV in NHP. Concentrated *E. coli* O86a:K61 supernatant (500-fold) was prepared according to standard enzyme preparation described in text, and incubated at 80°C (Panel A) or 95°C (Panel B) for various times. After incubation, samples were assayed using *E. coli* protease activity assay versus FV. Activities were generated using the *E. coli* protease standard curve (Fig. 5). n=2.

A)


% Protease Activity versus Unheated
160
140
120
100
80
60
40
20
0
Unheated
10 min
30min
1hour

B)

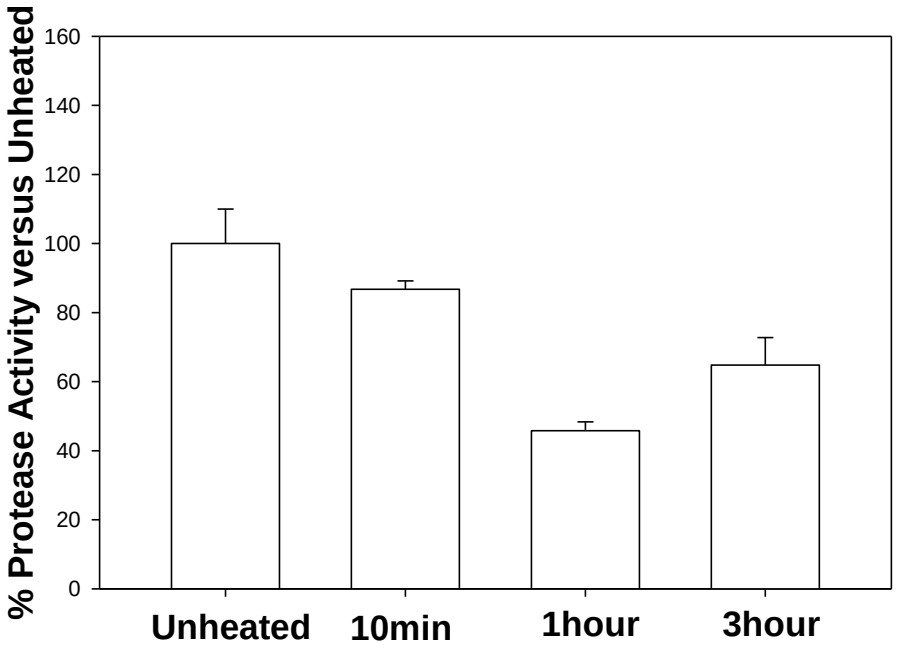

% Protease Activity versus Unheated
160
140
120
100
80
60
40
20
0
Unheated
10min
1hour
3hour

Figure 14.

Figure 15 Sephadex G-100 Gel Filtration Chromatography of *E. coli* Concentrated and Ultracentrifuged Culture Supernatant. Ultracentrifuged supernatant (600µl) was applied to a Sephadex G-100 column, and eluted in the same buffer as the sample (20mM Hepes, 150mM NaCl, 0.5M urea, 0.01% TritonX-100, 0.01% β-mercaptoethanol) at room temperature collecting 500µl fractions with a flow rate of 0.25ml/min. Protein concentrations (Circles) were calculated using the a280nm method described in text. *E. coli* protease activities versus FV in plasma (Squares) were calculated as described in text and compared to a protease standard curve (Fig. 5). See Materials and Methods for complete description of Sephadex G-100 gel filtration protocol.

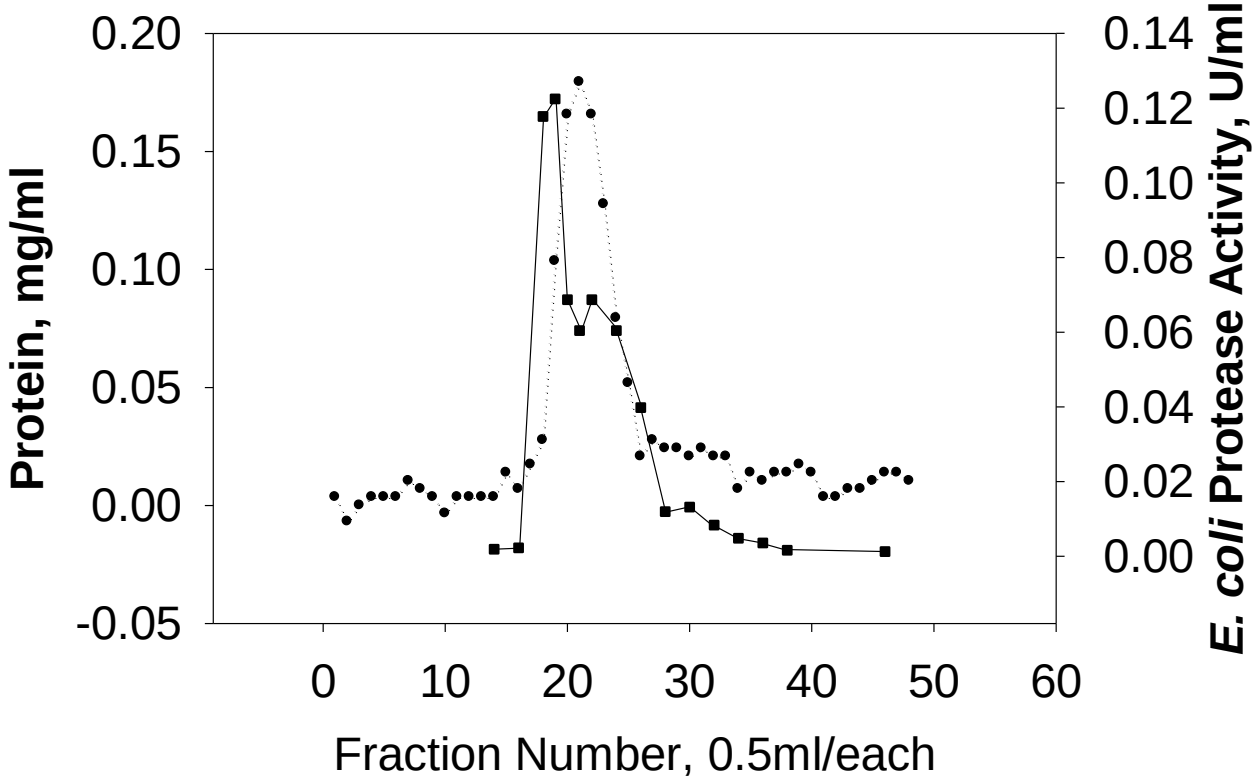

0.20
0.15
0.10
0.05
0.00
-0.05
Protein, mg/ml
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
E. coli Protease Activity, U/ml
0
10
20
30
40
50
60
Fraction Number, 0.5ml/each

Figure 15.

Figure 16 SDS-PAGE with Coomassie Brilliant Blue Staining of Protein Purification by TFF Filtration, Ultracentrifuge Separation, and Sephadex G-100 Column Chromatography. TFF Filtrate refers to TFF 10kDa MWCO concentrated filtrate after TFF 100kDa MWCO concentration. Ultracentrifuge pellet refers to the material that formed a pellet after 150,000 x g for 1.5h at 4°C. Ultracentrifuge supernatant is the material that remained soluble after ultracentrifugation. Ultracentrifuge supernatant was concentrated using CFF with a 10kDa MWCO filter, and applied to a Sephadex G-100 column (40ml bed volume). F14 (Fraction 14)-F38 (Fraction 38) are column elution fractions. See Figure 15 for complete column elution profile. Each sample (25µl) was loaded per well of a 4-20% polyacrylamide gradient gel, and electrophoresed at 150V, constant voltage. The gel was stained with 0.01% Coomassie Brilliant Blue, and photographed. See Materials and Methods for complete description of SDS-PAGE protocol.

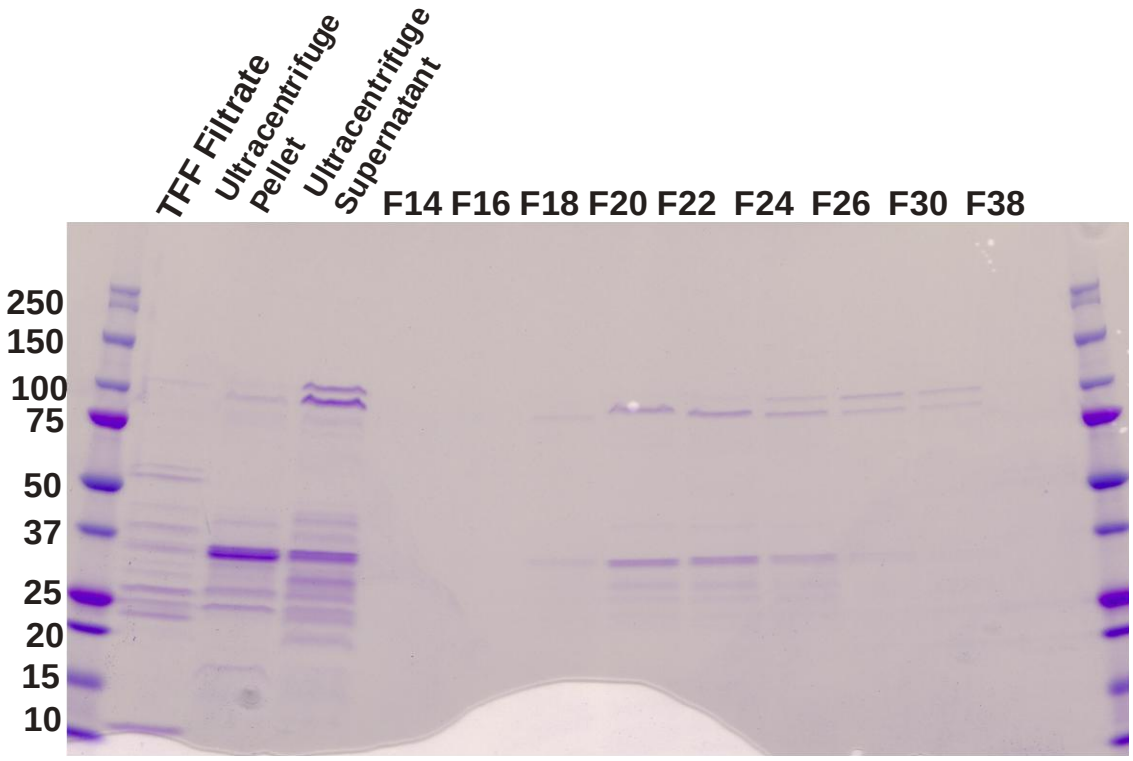

TFF Filtrate
Ultracentrifuge Pellet
Ultracentrifuge Supernatant
F14 F16 F18 F20 F22 F24 F26 F30 F38
250
150
100
75
50
37
25
20
15
10

Figure 16.

Figure 17 SDS-PAGE with Silver Staining of Protein Purification by TFF Filtration, Ultracentrifuge Separation, and Sephadex G-100 Column Chromatography. TFF Filtrate refers to TFF 10kDa MWCO concentrated filtrate after TFF 100kDa MWCO concentration. Ultracentrifuge pellet refers to the material that formed a pellet after 150,000 x g for 1.5h at 4°C. Ultracentrifuge supernatant is the material that remained soluble after ultracentrifugation. Ultracentrifuge supernatant was concentrated using CFF with a 10kDa MWCO filter, and applied to a Sephadex G-100 column (40ml bed volumn). F14 (Fraction 14)-F38 (Fraction 38) are column elution fractions. See Figure 15 for complete column elution profile. Each sample (25µl) was loaded per well of a 4-20% polyacrylamide gradient gel and electrophoresed at 150V, constant voltage. The gel was stained silver (Mullin et al 1981), and photographed. See Materials and Methods for complete description of SDS-PAGE and silver staining protocol. (Samples are the same as Figure 16)

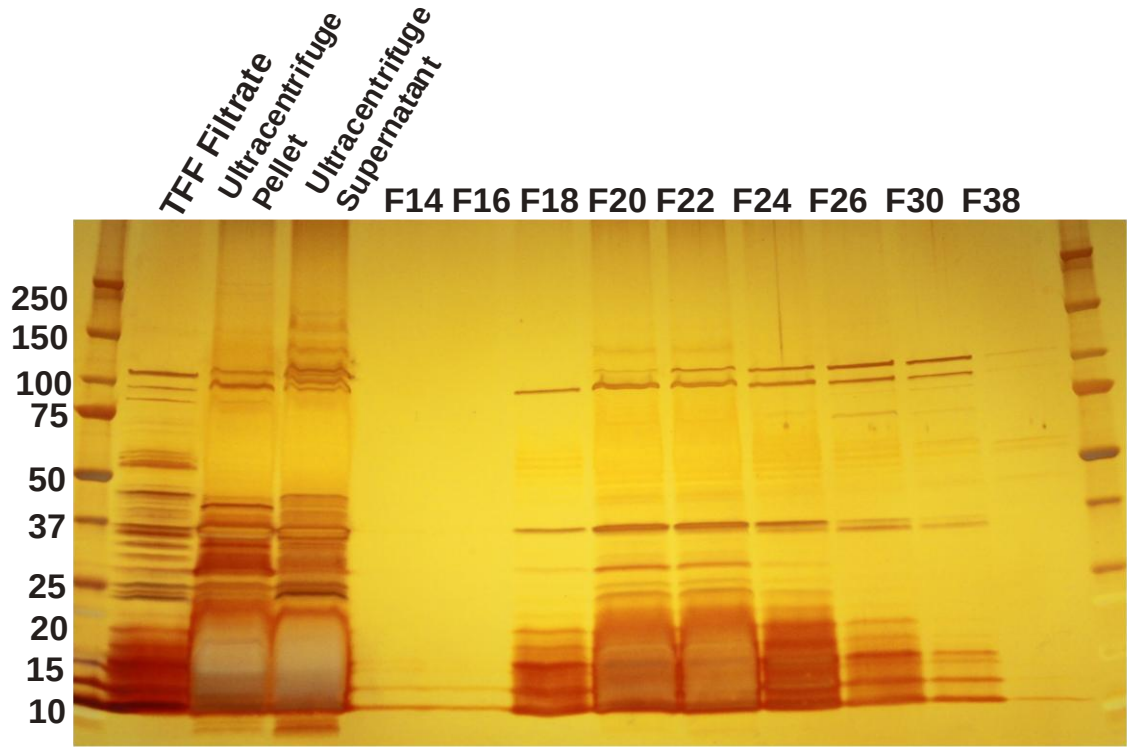

TFF Filtrate
Ultracentrifuge Pellet
Ultracentrifuge Supernatant
F14 F16 F18 F20 F22 F24 F26 F30 F38
250
150
100
75
50
37
25
20
15
10

Figure 17.

The non-symmetrical elution peak of activity may correlate with the presence of a third protein, seen in fraction 22 (and subsequent fractions) with an apparent molecular weight of approximately 110kDa. Analysis by SDS-PAGE with Silver staining (Figure 17) revealed large blurry bands in the 10-25kDa region of the gel and a distinct banding ladder in the 37-75kDa region, which may correlate to rough lipopolysacharide (LPS) or intact O-antigen of LPS, respectively, reported by others (Haurat et al 2010). LPS will stain with silver, but not by CBB staining (Haurat et al 2010). The purification table (Table 2) shows an approximate 1000-fold increase in specific activity compared to cell-free culture supernatant, with specific activity increases of 74-fold, 329-fold after TFF concentration, and ultracentrifugation, respectively. The protocol yielded approximately 8.5% of *E. coli* protease activity present in the TFF 100kDa retenate. The Sephadex G-100 gel filtration fractions which showed activity in the *E. coli* protease activity versus FV in NHP assay were subjected to Western blotting versus FV in NHP (Figure 18). A 10-fold dilution of the material applied to the column (Column SM; starting material; 10xdil), and all fractions which inactivated FV in the activity assay were also capable of cleaving intact FV (330kDa) to an inactive 250kDa product. Curiously, the 250kDa FV cleavage product produced by the *E. coli* protease cleavage is more intense than the intact 330kDa FV molecule.

To better understand the unusual elution profile from the Sephadex G-100 gel filtration column, the ultracentrifuged pellet (Containing 45% of the activity seen in the ultracentrifuge supernatant used in the column) was analyzed using negative staining (2% uranyl acetate) and transmission electron microscopy (Figure 19). A total of 9 images were analyzed to determine the size of the vesicles (n=2048) which were heterogeneous in nature. The maximum diameter of OMVs observed was 158nm, which is below the theoretical maximum of 200nm imposed by the 0.2µm filtration step used to remove any whole cells remaining after centrifugation of the bacterial culture. It is possible that *E. coli* O86a:K61 secretes larger OMVs; however, the protocol used here would not allow for their detection. The

Table 2 Purification Table of Protein Isolation Procedure. Culture supernatant refers to cell-free culture supernatant described in text. TFF 100kDa retenate refers to TFF 100kDa MWCO filter concentrated material and centrifugal filter flow 100kDa MWCO filter concentrated material (Total concentration 1L to 5ml). Ultracentrifugation refers to CFF 10kDa MWCO filter concentrated ultracentrifuge supernatant (5ml to 1ml). Gel Filtration refers to Sephadex G-100 gel filtration elution fractions with the highest *E. coli* protease-specific activities which were pooled and concentrated using CFF 10kDa MWCO filter. Activities were determined using *E. coli* protease activity assay versus FV and an *E. coli* protease standard curve (Fig. 3). Protein concentration was determined using a280nm method described in text.

	Total Activity, U	**Specific Activity, U/mg**	**Fold Purification**
Culture Supernatant	900	45	N/A
TFF 100kDa Retenate	1380	335.7	74
Ultra-centrifugation	1000	1490	329
Gel Filtration	117	4333	956

Table 2.

Figure 18 Western Blot for FV of Sephadex G-100 Gel Filtration Fractions. NHP refers to HBS treated plasma. F17 (Fraction 17)-F26 (Fraction 26) are Sephadex G-100 gel filtration eluted fractions (See Figure 14). Column SM (Starting material; 10x dil) is a 10-fold dilution of the material that was applied to the column, which was concentrated ultracentrifuged supernatant. Plasma (0.25µl) was loaded per well in 4-20% polyacrylamide gradient gels and electrophoresed for 1.5h at 150V constant voltage. Proteins were electroblotted onto a PVDF membrane for 16 hours at 35V constant voltage and were probed with sheep anti-human Factor V IgG and donkey anti-sheep IgG with conjugated HRP. See materials and methods for complete description of Western Blotting protocol. “NHP” refers to plasma which was treated with buffer alone. 250 refers to the migration distance of the 250kDa molecular weight standard.

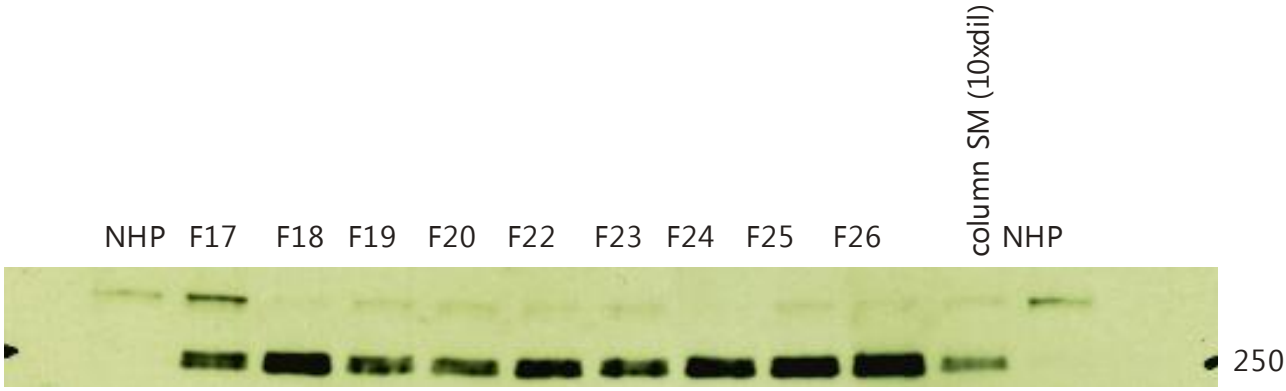

NHP
F17
F18
F19
F20
F22
F23
F24
F25
F26
column SM (10xdil)
NHP
250

Figure 18.

Figure 19 Transmission Electron Micrographs of Negative Stained *E. coli* O86a: K61 Outer Membrane Vesicles. OMVs were isolated as described in the text and negatively stained with 2% uranyl acetate at the Hospital for Sick Children (Toronto, ON). Panel A depicts 4000-fold concentrated OMVs at 62,000 x magnification. Panel B depicts 4000-fold concentrated OMVs at 120,000 x magnification. Panel C and D are 400-fold concentrated OMVs at 150,000 x magnification. Scale bars represent 100nm.

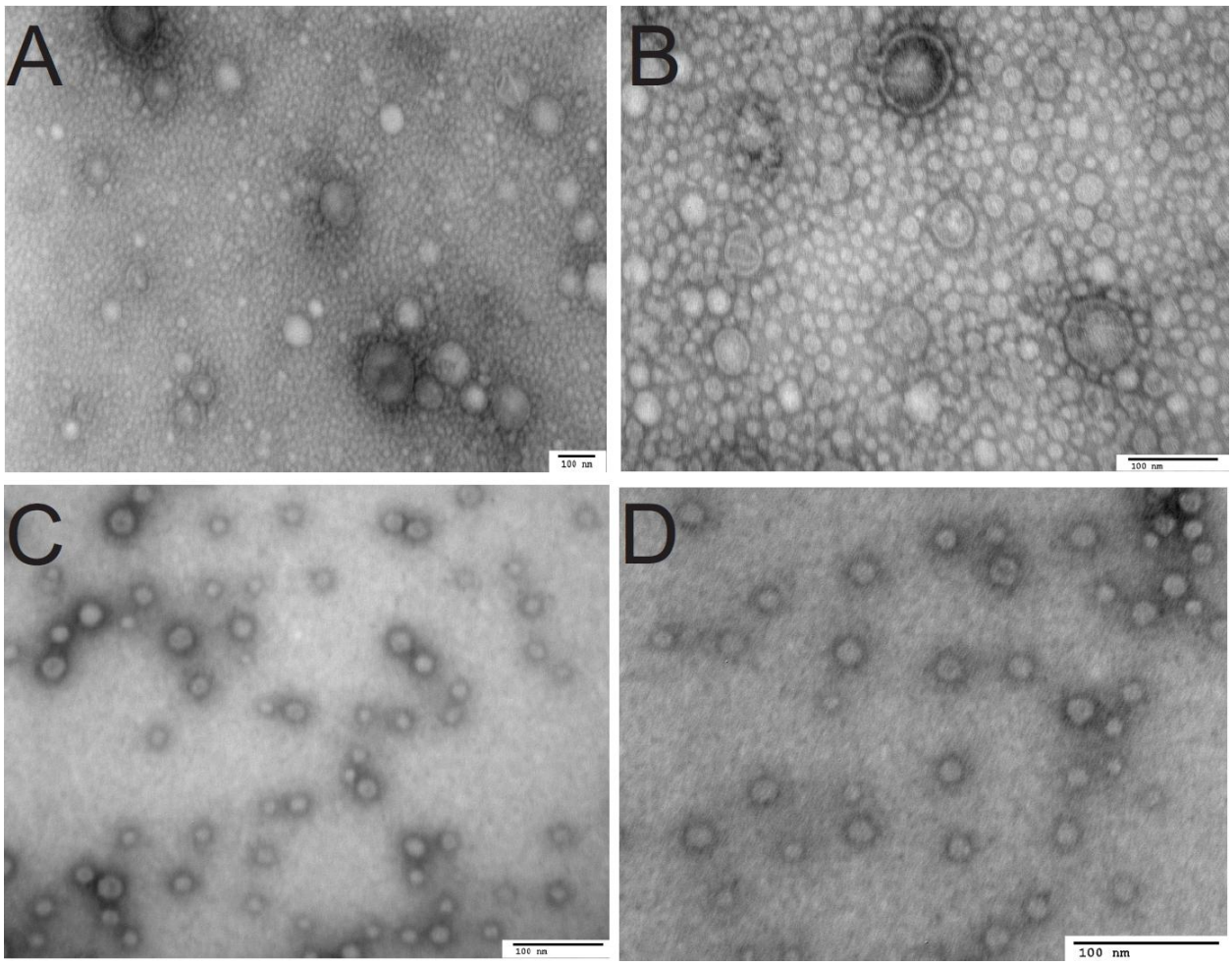


Figure 19.

minimum diameter of OMVs observed was 8nm, which is smaller than the expected 20-250nm range reported by others (Kulp and Kuehn 2010). However, OMVs of 8nm diameter would be expected to be retained by the approximately 5nm pore size of a 100kDa filter. The average diameter of the OMVs was (Mean ± Standard Deviation) 21nm ± 13nm. Figure 19, Panel A, depicts several large vesicles, with smaller vesicles constituting the majority of the background (62,000x magnification). Panel B shows a close-up of the smaller vesicles seen in Panel A (120,000x magnification). To ensure the smaller vesicles were not artefactual, a 100-fold dilution was made to allow for the detection of background grid staining (Panel C and D). These images show smaller vesicles in contrast to the background grid (150,000x magnification).

In an attempt to further purify the *E. coli* protease from the OMVs, various detergents, reducing agents, and solubilising reagents were screened for their effect on the 1-stage FV activity assay, and on the *E. coli* protease activity (Table 3). Although the 1-stage FV activity assay could tolerate concentrations above the critical micelle forming concentration (Cmc) of several detergents, the *E. coli* protease would not tolerate detergent concentrations at or above the Cmc of any detergent. This implies that the protease may require intact OMVs for function. Curiously, reducing agents such as β-mercaptoethanol and DTT significantly enhanced the protease activity (at 0.1%) by up to 500-fold, which would be consistent with the secreted *E. coli* protease belonging to a cysteine protease family (Yang et al 2011). However, as discussed above, the *E. coli* protease was not inhibited by cysteine protease inhibitors. Urea also enhanced activity by 2-fold at concentrations up to 1M; however, the FV activity assay would not tolerate urea concentrations in excess of 1M.

The effects of detergent solubilisation with 30mM CHAPS on protein binding to a Q-Sepharose anion exchange column was analyzed in Figure 20. Without a solubilising reagent, the proteins present in the *E. coli* supernatant would not bind to Q-Sepharose anion exchange columns at pH 7.4, and eluted immediately in fraction 1 (Panel B). The same effect was observed when the column was run at pH 8.0

Table 3 The Effects of Detergents on FV 1-stage Activity Assay and on *E. coli* Protease Activity. Various ionic and non-ionic detergents, reducing agents, and urea were screened for their effects on the 1-stage FV activity assay and the *E. coli* protease activity assay versus FV in NHP.

Reagent	Maximum Concentration of Reagent Tolerated by Activity Assay	Reduction in Protease Activity
Tween 20	1%	100%
Tween 80	1%	100%
TritonX-100	1%	100%
β-Mercaptoethanol	0.1%	~500-fold Activation
Urea	1M	~2-fold Activation
SDS	0.00001%	N/A
N-Lauroylsarcosine	<0.01%	N/A
Dithiothreitol (DTT)	0.1%	~500-fold Activation
CHAPS	10mM	100%

Table 3.

Figure 20 SDS-PAGE with Silver Staining of Fractions from Q-Sepharose Anion Exchange Chromatography. UC sup, refers to ultracentrifuge supernatant (See also in Fig. 16-17). Column SM depicts centrifugal filter flow 10kDa MWCO filter concentrated ultracentrifuged supernatant. F1 (Fraction 1) –F52 (Fraction 52) (Panel A) are eluted fractions. The column was run in 20mM Hepes pH 7.4 (Panel B), or with 20mM Hepes pH 7.4 with 30mM CHAPS (Panel A). Protein bound to the column in the presence of 30mM CHAPS (Panel A), but did not bind in the absence of 30mM CHAPS (Panel B). "+" indicates protease activity by *E. coli* protease activity assay versus FV, and "-" indicates no detectable activity. The binding buffer contained 30mM CHAPS, the elution buffer did not contain CHAPS (Panel A). Each sample (25µl) was loaded per well of a 4-20% polyacrylamide gradient gel and electrophoresed at 150V constant voltage. The gel was stained with silver (Mullin et al 1981) and photographed. See Materials and Methods for complete description of SDS-PAGE, and silver staining protocol. Molecular weight standard (Left and right of gel image) are in kDa.

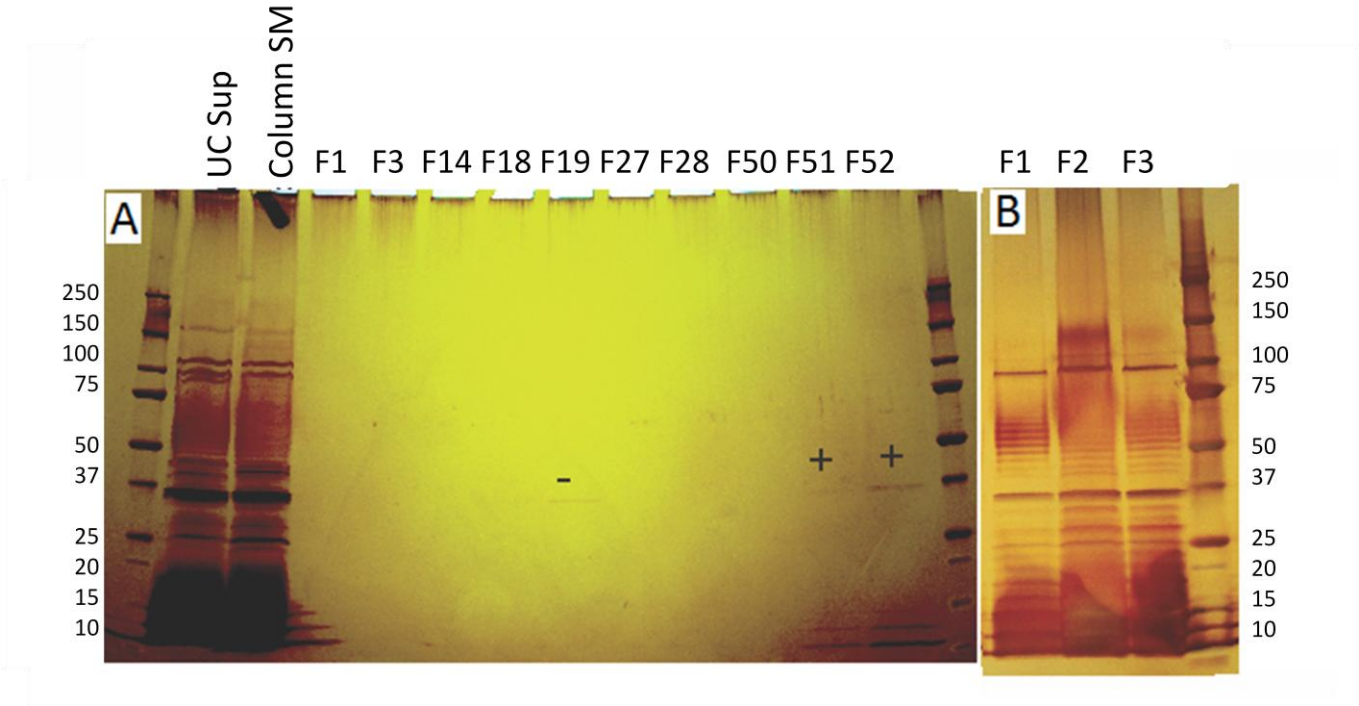

UC Sup
Column SM
F1
F3
F14
F18
F19
F27
F28
F50
F51
F52
F1
F2
F3
A
B
250
150
100
75
50
37
25
20
15
10
-
+
+

Figure 20.

(Data not shown). No protein or protease binding was observed with SP-Sephadex cation exchange chromatography at pH 5.5, 7, or 8 (Data not shown), or with Benzamidine Sepharose chromatography (Serine protease affinity column; data not shown). Solubilisation with 30mM CHAPS promoted protein binding to the column; however, 30mM CHAPS interfered with the FV activity assay, necessitating elution in the absence of CHAPS. A single protein eluted from the column in fraction 19 (At approximately 112.5mM NaCl) (Panel A), but no protease activity versus FV was associated with this eluted protein. A protein of the same molecular weight also eluted in fraction 51 and 52 at 1M NaCl, along with several low molecular weight species which were consistent with rough LPS. Fraction 51 and 52 demonstrated activity (Approximately 20% of the protease activity applied to the column) in the *E. coli* protease activity versus FV in human plasma assay; however, it is unclear if CHAPS was present in the sample, which would interfere with the measurement. Also, the NaCl concentration (1M) used to elute the protein from the Q-Sepharose bedding interfered with the activity assay; therefore, a correction value was used to estimate the protease activity. The majority of the protein which bound to the column did not elute after 1M NaCl wash and would not elute until the application of 70% ethanol; however, 70% ethanol is not compatible with any of the assays used in this study.

Given the inability to fractionate the protein sample beyond the conditions used in Table 2, the 3 major proteins which were associated with the OMVs that displayed protease activity versus FV in the microplate assay were N-Terminally sequenced (Table 4). Sequence data for the 90kDa and 110kDa protein could not be obtained due to insufficient protein concentration or the proteins being N-terminally blocked. The 37kDa protein generated sequencing data which was compared to the Swiss-Prot protein database. The DXVYNKDG (Where X is an unknown amino acid) sequence was reduced to VYNKDG (Most confidence), and compared with other known proteins of *E. coli*. Although a 6 amino acid sequence was insufficient to conclusively identify the sequenced protein, several candidate proteins have been identified. YedS (Theoretical protein), Outer membrane protein C (OmpC), Outer membrane

Table 4 N-Terminal Sequencing of Sephadex G-100 Gel Filtration Fractions with the Highest Protease Specific Activity. Purification table was described in Table 2 and SDS-PAGE was described in Figure 16-17. Sephadex G-100 fractions 17-19 were pooled and concentrated 8-fold by centrifugal flow filtration with a 10kDa MWCO filter. The samples were run on SDS-PAGE described in text and electroblotted onto PVDF membrane described in text. The samples were then sent for N-Terminal sequencing at the Hospital for Sick Children Advance Protein Technology Center (Toronto, ON). The 1 letter 8 amino acid sequence was modified to 6 amino acids and compared against the Swiss-Prot data base. X designates unknown amino acid. Bolded letters are “positive” hits, and underlined letters are “negative” hits. Numbers before or after the amino acid sequence refer to the amino acid position within the translated *E. coli* protein.

37kDa N-terminal Sequence: DXVYNKDG

BLAST Sequence: VYNKDG

Protein (Name)	Sequence	Identity (%)	Positives (%)	Function
YedS	$_{24}$VYNKDG$_{29}$	100	100	porin
OmpC	$_{24}$VYNKDG$_{29}$	100	100	porin
OmpN	$_{24}$VYNKDG$_{29}$	100	100	porin
YddL	$_{24}$VYNKDG$_{29}$	100	100	porin
OmpF	$_{25}$**I**YNKDG$_{30}$	83	100	porin
Pic	$_{86}$VY**D**KDG$_{91}$	83	100	serine protease
Pic	$_{854}$VYLTDG$_{859}$	67	67	serine protease
Hbp	$_{83}$**I**YNKQG$_{88}$	67	83	serine protease

Table 4.

protein N (OmpN), YddL(Theoretical protein), and Outer membrane protein F (OmpF) are all porins which have molecular weights that are consistent with the predicted molecular weight (37kDa) based on SDS-PAGE (Figure 13 and 14), and produced 100% positive sequence alignments. OMV associated porins have been described for other gastro-pathogenic bacteria (Mullaney et al 2009); however, they would not be expected to have proteolytic activity. Also identified was Pic, an *E. coli* SPATE, which other investigators have demonstrated has proteolytic activity towards FV (Dutta et al 2002); however, Pic has a molecular weight of >100kDa. Two separate regions within Pic produced positive alignments, one near the N-terminal end (100% positive), and one near the middle of the protein (67% positive). Pic has not been reported to associate with OMVs. Hemoglobin protease (Hbp), another *E. coli* SPATE, also showed some (67% positive) sequence alignment similarities; however, this protease has not been reported to cleave FV (Dautin 2010), nor has it been reported to associate with OMVs. It is currently unclear whether the 37kDa protein is in fact a porin, or a proteolytic breakdown product of Pic, or Hbp.

Discussion

The kinetic microplate-based assay outlined here describes the development of a new, fast, economical, and simple technique for measurement of FV coagulation activity in samples of human plasma which have or have not been intentionally activated by thrombin. Development of this assay was critical to the success of this study as it allowed for the quantification of *E. coli* protease activity against FV. The assay measures the increase in turbidity of plasma during clot formation at 405nm using a kinetic microplate-reader. The assay also has the added advantage of using small sample volumes (50µl) as well as being able to analyze multiple samples simultaneously (up to 12). These assay characteristics are advantageous when expensive reagents are required (Such as factor-deficient plasmas), or when only small sample volumes are available (Such as clinical patient plasmas). The assay's speed allows for the analysis of a large number of samples, which is necessary during FV protein purification procedures from human or animal plasma, or when purifying proteins which activate/inactivate FV coagulation activity.

The results indicate that the FV in the DIC patient plasmas was functionally less active than NHP because of prolonged clot times and decreased initial rates of clot formation in the FV 1-stage assay. However, the initial rates of clot formation in the FV 2-stage assay in the DIC patients were not largely different from NHP. The extents of clot formation in the DIC patient plasmas were also not largely different from NHP in the FV 1-stage and 2-stage assays. The unchanged extent of clot formation in the DIC patients may be attributed to non-FV coagulation factor levels, such as fibrinogen in the FV-deficient plasma and may be unrelated to characteristics of the patient plasmas.

The FV in the DIC patient plasmas supported both a delayed and slower rate of fibrin clot formation compared with NHP. Compared with NHP, the FV 1-stage, FV 2-stage, and total (FV 2-stage activity – FV 1-stage activity) activities in the DIC patient plasma were decreased on average by

approximately 54%, 44%, and 42%, respectively. The decreased FV 1-stage, 2-stage, and total activities may have been due to increased FV consumption (Mammen 2000) and/or inactivation (Samis et al 2004) which occurred during the pathogenesis of this acquired blood disorder in the patients studied. In previous studies with patient plasmas, it was demonstrated that DIC patient FV antigen levels were not significantly different from NHP (Samis 2011, unpublished observations); however, inactivation of FV by host and/or pathogen proteases may allow for increased epitope recognition by antibodies in the absence of functional FV activity.

The results of the microplate assay indicate that measurement of FV coagulation activity in human plasma samples may be obtained from the time and initial rate measurements of clot formation from the FV 1-stage assay, and the time of clot formation and total activity measurement from the FV 2-stage assay. It was, however, not possible to obtain a quantitative measurement of FV coagulation activity in a plasma sample based on the extent of clot formation in the FV 1- and 2-stage assay, or the initial rate of clot formation in the FV 2-stage assay. During the production of a typical FV activity standard curve, the extent of clot formation decreases initially upon serial dilution of NHP; however, subsequent dilutions of NHP result in an increase in the extent of clot formation. Initially, the decrease in extent may be attributed to decreasing levels of a FV activator of this process in NHP. The fact that the extent of clot formation increases after further dilution of NHP may represent the real effect of decreasing FV concentrations on clot formation and/or dilution of a FV inhibitor of this process in NHP. This increase in extent of clot formation as FV coagulation activity decreases may be a compensation mechanism employed by the host in the event of FV consumption or inactivation during pathological states; however, further studies are needed to confirm these speculations.

E. coli represents a major health threat despite being the most heavily studied life form, and is one of the most important clinically relevant pathogens. *E. coli* is the second most frequently isolated

pathogen from bloodstream infections (Kern 2011) accounts for up to 9% of ventilator associated pneumonia episodes in Canada (Cook et al 1998), 8% of surgical site infections in the US (National Nosocomial Infections Surveillance System, 1996), and over 850,000 deaths per annum globally (Russo and Johnson, 2003). Despite intensified prevention efforts, *E. coli* remains a persistent pathogen in the US and around the world (Nyachuba, 2010). Traditionally, *E. coli* infections have been successfully treated using antimicrobial therapy; however, *E. coli* strains that are resistant to classical (Johnson et al 2005), and modern (Kern 2011) antibiotics are on the rise. Anti-virulence therapy represents a promising new approach to fighting infection, as it removes heavy selective pressures to adapt from the pathogen. Virulence factors that target the coagulation system are extremely important during infection. The most striking evidence of the importance of virulence factors directed at the coagulation system comes from *Yersinia pestis*. The loss of the plasminogen activating protease, Pla results in a million-fold increase in the median lethal dose of bacteria in mice (Sodeinde et al 1992). The importance of *E. coli* virulence factors targeted at the coagulation system requires further investigation.

Immunoblotting of 6 clinical *E. coli* isolates, *E. coli* O86a: K61, and *E. coli* JM109 concentrated culture supernatants demonstrates the prevalence of proteases capable of cleaving and inactivating FV amongst pathogenic and non-pathogenic *E. coli* strains. Approximately 1/3 of the clinical isolates secreted proteases that were retained by a 100kDa filter, and were capable of cleaving and inactivating FV in NHP. These results show that some, but not all pathogenic *E. coli* strains produce large proteases capable of cleaving FV. Others have shown that *E. coli* secretes large proteases capable of cleaving FV (Brunder et al 1997); however, no effect on FV activity was measured. Further study is required to elucidate whether smaller secreted proteases are present in the culture supernatants of clinical isolates which did not proteolytically degrade and inactivate FV. *E coli* O86a: K61, the strain used by Samis et al 2009 to study sepsis in a baboon model and the strain used in this study, secreted a protease which was capable of cleaving FV to a 250kDa partially/inactive product. The molecular weight (250kDa) of the

proteolytic cleavage product of FV in the baboon plasma was consistent with the cleavage product produced by the *E. coli* protease studied here, indicating the *E. coli* protease was likely responsible for FV cleavage and inactivation during experimental sepsis. Direct inactivation of purified FV (Data not shown) by the *E. coli* O86a:K61 secreted protease suggests that host proteases such as plasmin(ogen), which has been shown to be activated by *E. coli* proteases (Lundrigan and Webb, 1992), and also generates a similar sized FV cleavage product (Samis, unpublished results) are not responsible for the inactivation event. Although plasmin is a candidate host protease which is capable of cleaving and inactivating FVa, it has been demonstrated that plasmin activates the procofactor FV molecule after 30 minute incubations in comparable activity assays (Lee and Mann 1989), which is inconsistent with results obtained in this study. The non-pathogenic laboratory *E. coli* strain JM109 also generated a FV cleavage product of 250kDa. Others have demonstrated that minor modifications to non-virulent housekeeping proteases may result in the generation of potent virulence factors (Kukkonen et al 2001). Further study is necessary to determine the virulent nature of the secreted *E. coli* JM109 protease. Nevertheless, the JM109 strain may be ideal as a model for studying proteolytic inactivation of FV because the strain is not pathogenic to humans.

FV cleavage by the secreted *E. coli* O86a: K61 protease was also associated with decreased FV coagulation activity in the FV 1-stage microplate assay. Complete cleavage of the intact 330kDa human FV molecule to a 250kDa product was associated with an 80% decrease in FV 1-stage activity. These results are consistent with the results obtained by Samis et al 2009 in non-lethal and lethal experimental sepsis in baboons, where complete cleavage of FV to a 250kDa product was associated with an 80% decrease in activity. The results suggest the 250kDa FV cleavage product generated by the secreted *E. coli* protease retains approximately 20% of the functional activity of the intact 330kDa procofactor. However, it is unclear whether the 250kDa product is still active, or if the 330kDa intact species is present at a concentration which is below the detection limit of the assay. Isolation of the 250kDa

cleavage product would be required to clarify the functional FV activity of this proteolytic product in a system of purified components.

The inactivation of FV coagulation activity by the *E. coli* O86a: K61 protease also resulted in an increase in the extent of clotting. As discussed earlier, it is unclear whether this was the result of FV inactivation. It is possible that inactivation of a host protease by TFPI, which is responsible for "shutting down" coagulation, would generate this effect. TFPI, which would be expected to be present in NHP during tissue factor induced coagulation in the FV 1-stage activity assay, may also be inactivated by the *E. coli* protease. Other *E. coli* proteases have been shown to inactivate TFPI (Yun et al 2009); however, further study is necessary to confirm whether TFPI is a target of the *E. coli* protease in this study. Curiously, when the *E. coli* protease was incubated with NHP and coagulation was induced via the Contact pathway (aPTT assay), there was a decrease in the extent of clotting compared to controls (Data not shown). All other regulators of coagulation would be expected to effect the clotting event regardless of which pathway was used to induce clotting.

The results indicate that the rate of clot formation remained unchanged in samples that were treated with the *E. coli* protease compared to samples treated with buffer. As indicated earlier, FV levels influenced the rate of clot formation. It is possible that the 250kDa cleavage product may still participate in the clot formation event in some way, and therefore has no effect on the rate of clot formation. Nevertheless, this result remains unclear. It is, however, clear that the *E. coli* protease increased the time for clot formation, and the resulting clot produced had an increased extent of clot formation compared to samples treated with buffer alone.

The results indicate that when NHP is incubated with the *E. coli* protease, there was a prolongation in the aPTT time compared to samples treated with buffer. FV inactivation would be expected to prolong the time for clot formation in both TF-induced (FV 1-stage/*E. coli* protease activity

assay) and Contact pathway-induced (aPPT), as individuals deficient in FV present with prolonged PT and aPPT clot times (Huang and Keorper 2008). Unlike the FV 1-stage assay, the aPPT assay does not distinguish between which coagulation factors in the Contact/Common pathway have been inactivated, and it is therefore unclear whether other coagulation factors besides FV are inactivated by the *E. coli* protease. Altering the deficient plasma and the reagent used to initiate clotting in the FV 1-stage assay would allow for detection of protease-dependent inactivation events of other coagulation factors in future studies.

The results indicate that when whole human blood was incubated with the *E. coli* protease, there was a prolongation in the time for TF-induced clot formation compared to samples treated with buffer. Automated whole blood coagulation assays have been used to evaluate coagulopathy (Larsen et al 2011), and are commonly used for monitoring coagulation during surgery (Bowers and Ferguson, 1993). A modified version of the whole blood assay used in this study was able to detect activation of FV and/or FVIII (Kenichi et al 2010); however, the ability of the assay to detect factor V deficiency or inactivation has not been reported. Nevertheless, the prolongation in the time for clot formation when cellular components are present is consistent with bacterial secretions being responsible for the hemorrhagic diarrhea observed in the child that the *E. coli* strain was isolated from. Therefore, this result would be expected after proteolytic inactivation of FV by the *E. coli* protease. Further investigation is required to analyze inactivation of other coagulation factors that may contribute to the prolongation of clot time in the whole blood clotting assay.

The results indicate that the *E. coli* protease was inhibited modestly by PMSF (Approximately 20%) and almost completely (Approximately 90%) by A1PI. PMSF irreversibly inhibits serine proteases such as trypsin and thrombin by sulfonating the hydroxyl group of the serine residue at the active site (Gold 1967). PMSF is also capable of inhibiting some cysteine proteases (Scopes 1994). PMSF is an ideal

serine protease inhibitor for this study because of its relatively short half-life when dissolved in water. PMSF has a half-life of approximately 35 minutes in aqueous solutions of neutral pH at room temperature and is almost completely inactivated after 1 hour at room temperature (James 1977). These characteristics of irreversible inhibition and short half-life allow for experimental conditions where inhibition of the *E. coli* protease may be monitored without inhibiting the serine proteases of the coagulation cascade. The *E. coli* protease activity was reduced by approximately 20% after incubation with 1.5x the maximum suggested concentration of PMSF by the manufacturer (Sigma, Oakville, ON). This suggests, but does not prove that the *E. coli* protease is a serine protease. Other non-serine proteases of *E. coli* have been inhibited by serine protease inhibitors (Mangel et al 1994). The *E. coli* protease was not inhibited by E64 or iodoacetamide which suggests that the protease is not a cysteine protease. The *E. coli* protease was also resistant to the antimicrobial tetracycline, which is a mild metal ion chelator which has been shown by others to inhibit metallo-proteases (Imamura et al 2001). Tetracycline was used to limit any effects on the coagulation proteases which also depend on metal ions (Ca^{2+}) for functional activity. Tri-sodium citrate and EDTA are more commonly used to demonstrate that a protease is metal ion dependant; however, they were used to prevent coagulation during the blood drawing process and for the generation of FV-deficient plasma, respectively, and would therefore not be ideal for use in this study. The concentration of tetracycline incubated with the *E. coli* protease was comparable to the concentrations used by Imamura et al 2001, which suggests the *E. coli* protease is not metal ion-dependant. However, given the metal-ion dependant nature of FV and the coagulation cascade, an alternative substrate may be required to conclusively demonstrate the *E. coli* protease is not metal ion dependant.

A1PI, commonly referred to as alpha 1-antitrypsin, is a plasma derived polypeptide which inhibits a broad range of human proteases, though human neutrophil elastase is considered its primary target (Kalsheker, 1988). A1PI inhibited the *E. coli* protease activity against FV by 90% at physiological

concentrations (1.5mg/ml) (Normal range: 1.5-3.1mg/ml (Deam et al 1989)). The plasma used during standard assaying of the *E. coli* protease activity was diluted 5-fold before it was incubated with an equal volume of the *E. coli* protease, and would therefore be expected to contain approximately 0.15-0.31mg/ml A1PI, which may reduce the protease activity during Western blotting and the *E. coli* activity versus FV in human plasma assay. A1PI deficiency is one of the most common inherited disorders in Caucasians (Kalsheker, 1988). Individuals who are deficient in A1PI are at an increased risk of lung infection by opportunistic pathogens (Chan et al 2007). A1PI levels increased in response to *E. coli* O86a: K61 during experimental sepsis in baboons, but was inactivated during the later stages of infection (Samis et al 2009). A1PI has been shown to be inactivated by bacterial enzymes (Padrines and Bieth 1989) and bacterial toxins which produce anti-coagulant effects (Nlclasen et al 2011). The potent inactivation of the *E. coli* protease by A1PI may support further study into its potential use as a therapeutic agent during *E. coli* infections.

The results indicated that the *E. coli* protease activity versus FV was inhibited by Zn(II) and Cu(II), but not Fe (II). Others have reported that OmpT, a membrane bound *E. coli* aspartyl protease which activates plasminogen, is inhibited by divalent cations (Sugimura and Nishihara 1988). Zn^{2+} has been shown to inhibit aspartyl proteases by binding directly to the active aspartyl residue (York et al 1993), which suggests the *E. coli* protease may be an aspartyl protease. Cu^{2+} inhibited the *E. coli* protease versus FV, which is interesting because Cu^{2+} has also been shown to reduce A1PI inhibition towards trypsin and human neutrophil elastase (Kwon et al 1990), and A1PI was shown here to inhibit the *E. coli* protease. Fe^{2+} did not inhibit the *E. coli* protease activity versus FV at the concentrations tested. Others have demonstrated that proteases of *E. coli* which are capable of cleaving FV are also involved in iron acquisition during pathogenesis (Boisen 2009), which may be the case for the *E. coli* protease analyzed in this study.

The *E. coli* protease showed remarkable thermal stability, retaining over 50% of its activity after 3 hour incubations at 95°C. The thermal stability of the *E. coli* protease may be a result of its association with OMVs, which have been shown to stabilize their protein cargo (Kulp and Kuehn 2010). Secreted bacterial proteins must be resistant to stresses because they are exposed to harsh environments; however, the thermal stability of this protease is unusual. Other *E. coli* proteases have been shown to be incredibly heat stable, such as the plasminogen activator OmpT, which is routinely boiled to allow for re-folding during purification (Manel et al 1994). The thermal stability of the protease may be related to its ability to survive elevated temperatures in the host during infection, as other *E. coli* virulence factors have been reported to be heat stable (Lortie et al 1991).

The results of this study indicate that *E. coli* O86a: K61 secretes OMVs. Although others have theorized that nearly all gram-negative bacteria secrete OMVs during their lifecycles (Ellis and Kuehn 2010), the ability of EPEC strains to produce OMVs has not been reported. However, enterohaemorrhagic (EHEC) (Wai et al 2003), enterotoxigenic (ETEC) (Horstman and Kuehn, 2000), Shiga toxin producing (STEC) (Yokoyama et al 2000), uropathogenic (UPEC) (Kouokam et al 2006), and extraintestinal (ExPEC) (Scorza et al 2008) pathogenic *E. coli* strains have been shown to produce OMVs. OMV interactions with the coagulation system have not been reported. Transmission electron micrographs clearly demonstrate that *E. coli* O86a: K61 secretes OMVs which are heterogeneous in nature. Identification of the proteins which associate with these OMVs is of critical importance in order to understand their role(s) in deregulating the coagulation system through inactivation FV, as well as their role(s) in bacterial pathogenesis as a whole.

Purification and isolation attempts of the *E. coli* protease from the OMVs has proved challenging, as the secreted protease did not fractionate from lipid using commonly used protocols such as ammonium sulphate precipitation, polyethylene glycol precipitation, Q-Sepharose anion exchange

chromatography (Over broad pH ranges), SP-Sephadex cation exchange chromatography (Over broad pH range), Benzamidine Sepharose serine protease affinity chromatography, or sucrose gradient ultracentrifugation. Although the *E. coli* protease was not purified to complete homogeneity, approximately 1000-fold purification was achieved from the initial culture supernatant using TFF filtration, ultracentrifugation, and Sephadex G-100 gel filtration chromatography. The inability to achieve protein fractionation and purification of the *E. coli* protease using precipitation techniques was likely due to the presence of OMVs, which have been reported to interfere with standard precipitation protocols (Kulp and Kuehn 2010). OMVs would also be expected to interfere with affinity based chromatography (Including ion exchange) by sterically restricting access of the protein to the functional groups on the column beads. Also, the size of the OMV may have prevented diffusion into the beads containing the functional groups. However, the purification methods in this study are comparable to methods used by others to isolate OMVs (Kulp and Kuehn 2010). The *E. coli* protease activity eluted with two proteins with molecular weights of 37kDa and 90kDa early on during Sephadex G-100 gel filtration chromatography, suggesting its association with the larger OMVs observed. It remains unclear whether these two proteins are related or different, as the 37kDa band may be a proteolytic breakdown product of the 90kDa species, and several *E. coli* proteases have been shown to auto-proteolytically degrade during secretion and purification, including several SPATEs which have been shown to cleave FV (Dutta et al 2002). The non-symmetrical nature of the eluting protease activity from Sephadex G-100 gel filtration chromatography may be the result of a 3rd (110kDa) species. The large apparent molecular weight of two of the secreted proteins would be consistent with other reported SPATEs, and it is not unusual to have multiple SPATEs in one pathogenic *E. coli* strain (Dautin 2010).

The anti-coagulant nature of the OMVs is somewhat surprising given that they are rich in LPS, which is a known pro-coagulant (Pernerstorfer et al 1999). The fact that the OMV associated protease targets FV for cleavage and inactivation is not surprising, as individuals who are FV deficient most often

present with bleeding episodes in skin, mucosa, and gastrointestinal tract (Huang and Koerper 2008). Although EPEC strains such as O86a: K61 do not typically enter the bloodstream, the OMVs may be secreted into the bloodstream after the development of an EPEC associated lesion within the gut. The anticoagulant nature of the OMVs would allow for entry through the developing fibrin clot at the site of infection. Once access to the bloodstream is obtained it is critical for the host to prevent the dissemination of the OMVs, as they are extremely virulent in nature and may cause mortality at very low concentrations in mice (Park et al 2010). EPEC strains may benefit from the generation of a net bleeding tendency in the host, as this would provide access to the bloodstream and iron. EPEC strains have been reported with virulence factors which are capable of lysing red blood cells for the acquisition of iron (Larzabal et al 2010), a critical micronutrient required during their pathogenesis in hosts (Skaar 2010; Wooldridge and Williams 1993). Further study is required to determine whether the OMVs produced by *E. coli* O86a: K61 enter the bloodstream or whether they localize at the site of the EPEC lesion to promote bleeding.

Further attempts to purify the secreted *E. coli* protease from OMVs have proven to be difficult. Disruption of the OMVs by detergents and solubilising reagents leads to immediate loss of protease activity. However, some solubilising reagents such as urea (1M) and β-mercaptoethanol (0.1%) increased protease activity towards FV by 2 fold, and up to 500 fold, respectively. The 37kDa species was purified to apparent homogeneity after solubilisation with 30mM CHAPS and Q-Sepharose anion exchange chromatography; however, attempts to reconstitute the protease activity towards FV were unsuccessful. Curiously, a protein of the same apparent molecular weight eluted later in the NaCl gradient with what appeared to be rough LPS by silver staining. These fractions possessed protease activity against FV in NHP.

Several characteristics of the *E. coli* protease from this study are consistent with it being OmpT, a 35kDa membrane bound aspartyl protease of *E. coli* which requires rough LPS for proteolytic function (Hwang et al 2007). OmpT is mildly inhibited by serine protease inhibitors, and may survive temperatures up to 95°C (Mangel et al 1994). OmpT is also inhibited by Zn^{2+} and Cu^{2+} (Sugimura and Nishihara 1988). OmpT retains proteolytic activity at up to 4M urea, and prefers denatured substrates (White et al 1995). OmpT is also expressed by *E. coli* K-12 strains (Hritonenko and Stathopoulos 2007), and would therefore be present in *E. coli* JM109. It is possible that OmpT or a related protease is being secreted into culture media via the OMVs; however, this has not been reported by others.

N-terminal sequencing of the 3 main proteins from *E. coli* OMVs was perfomed to identify the protease. N-terminal sequencing of the 37kDa protein did not confirm the presence of OmpT. Instead, several porins and two SPATEs displayed significant amino acid homology to the N-terminal sequence obtained. YedS , OmpC, OmpN, YddL, and OmpF are porins of *E. coli* with approximate molecular weights which are within 5kDa of the predicted 37kDa molecular weight shown by SDS-PAGE, and showed 100% positive matches to the basic local alignment search tool (BLAST) sequence. Porins have been reported to associate with OMVs of other gastro-pathogens (Mullaney et al 2009); however, proteolytic activity has not been reported with these proteins. Pic, an *E. coli* SPATE which has been demonstrated to cleave FV, has two distinct regions with homology (100% and 67%) to the sequence obtained by N-terminal sequencing, at residues 86-91 and 854-859, respectively. Pic is translated as a 146kDa polypeptide, which undergoes proteolytic processing during secretion. The active secreted form is 109.4kDa (Henderson et al 1999). N-terminal sequencing data obtained in this study was inconsistent with others who demonstrated N-terminal sequencing, starting at G^{56} and N^{1096} for the secreted and membrane bound component, respectively (Henderson et al 1999). However, it is possible that the intact Pic protease was proteolytically degraded, and N-terminal sequencing data was obtained from a cleavage product. Pic's role as a virulence factor remains somewhat unclear, but it is capable of cleaving

FV and haemoglobin (Boisen et al 2009). Pic also provides serum resistance, allowing bacterial growth in serum of non-pathogenic bacteria which would otherwise be killed. Others have speculated that this is a result of inactivation of a complement factor (Henderson et al 1999), but complement factor inactivation has not been confirmed (Dautin 2010). Hbp, another *E. coli* SPATE, also showed homology (83%) to the N-terminal sequence obtained at residues 83-88, though again at a sight which would not be expected to be N-terminally exposed unless proteolytic degradation took place. Hbp, like Pic, proteolytically degrades haemoglobin; however, Hbp is also capable of binding heme, and therefore has been linked to iron acquisition (Otto et al 2002). Proteolytic processing of FV has not been reported with Hbp. Further sequence analysis of the 3 semi-purified *E. coli* proteins is required to conclusively determine their identity.

This study has demonstrated that a secreted *E. coli* O86a: K61 OMV associated protease behaves as a virulence factor that targets the coagulation system through inactivation of FV, to promote a bleeding tendency in infected hosts. The OMV associated *E. coli* protease, when treated with whole blood or blood plasma, significantly prolonged the time required for contact or tissue factor activated fibrin production. The *E. coli* OMV protease would be expected to be concentrated at or near the site of infection where significant FV inactivation would occur. Inactivation of FV would result in a fitness advantage by increasing the likelihood of bacterial survival and transmittance to new host through deregulation of the host immune response. Further study is required to determine whether the secreted *E. coli* OMV protease gains access to the bloodstream to promote further deregulation of the host's systems, or whether localization occurs at the site of the EPEC associated lesion to promote bleeding for the acquisition of nutrients.

In future research, a combination of tandem mass spectrometry protein sequencing, N-terminal protein sequencing, and PCR gene sequencing could be implemented to conclusively determine the

identities of all three proteins which associate with the OMVs. Detergent solubilisation and column chromatography could be implemented to purify the secreted *E. coli* OMV protease to homogeneity so that it may be further characterized biochemically. This may require the use of molecular genetic techniques to allow for overexpression of the *E. coli* protease. The effects of the purified *E. coli* OMV protease on other coagulation factors could also be examined. The effects of the purified protease on fibrin formation in human plasma and its effects of fibrin degradation in human plasma could also be explored. Using protease deletion constructs, the effects of the secreted *E. coli* OMV protease on bacterial growth *in vitro* and virulence towards animal cells/models *in vitro*/ *in vivo* may also be examined. Finally, the secreted *E. coli* OMV protease may represent a target for new diagnostics, therapeutics, anti-virulent drug design, and/or vaccines. Clearly, future research of the effects of the secreted *E. coli* OMV protease on the coagulation, immune, and complement systems is warranted to firmly establish its role as a virulence factor that enhances bacterial proliferation and transmission by deregulating host systems.

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