F-necrophorum Ecotin Inhibits Human Plasma Kallikrein and Human Neutrophil Elastase

Katie Wright
Pamela Greenwell
Patrick Kimmitt

Faculty of Science and Technology, University of Westminster

This is a copy of the poster presented at the 115th General meeting of the American Society of Microbiology, New Orleans, Louisiana, USA, 30 May ï 2 June 2015.

UNIVERSITY OF RIGOUR RESEARCH RESULTS WESTMINSTER

F. necrophorum Ecotin Inhibits Human Plasma Kallikrein and Human Neutrophil Elastase

Katie Wright, Pamela Greenwell and Patrick Kimmitt
Faculty of Science and Technology, University of Westminster, London, UK

SOCIETY FOR GENERAL MICROBIOLOGY

Introduction

Fusobacterium necrophorum is a causative agent of Lemierre's syndrome (LS) which is characterised by thrombophlebitis of the jugular vein and bacteraemia.[1,2] *F. necrophorum* is a Gram-negative, anaerobic bacterium known to possess virulence genes such as a haemolysin,[3] filamentous haemagglutinin[4] and leukotoxin,[5] which target host blood components. Ecotin is a serine protease inhibitor found in the periplasm of *Escherichia coli*. It has been shown to be a tight binding inhibitor of factor XIIa, plasma kallikrein and human leukocyte elastase and to have a potent anticoagulant effect on human plasma.[6,7] This has not previously been characterised in *F. necrophorum*.

Methods

PCR and Sanger sequencing were used to confirm the presence of the ecotin gene in the genomes of a collection of 26 *F. necrophorum* clinical and reference strains using the primers in Table 1.

Table 1: Primers used for PCR and Sanger sequencing.

Primer	Oligonucleotide sequence (5' - 3')
Ecotin_F	GGCAACCAAAGACATGTAGGG
Ecotin_R	GTACCACGAAACATGCATACTT

A plasmid insert of the gene was synthesised and ligated into a pET-16b vector. BL21(DE3) chemically competent *E. coli* cells were transformed with the plasmid and induced to express the protein with 0.5 mM IPTG. The histidine-tagged protein was purified under native conditions by immobilised metal affinity chromatography over a cross-linked agarose matrix charged with nickel ions.

Ecotin was added to human plasma kallikrein (HPK) and human neutrophil elastase (HNE) at concentrations of 0, 12.5, 25, 50 and 100 nM and incubated for 1 hour at room temperature to equilibrate. HPK and HNE substrates were added to their respective enzymes at concentrations of 0.015, 0.03, 0.06, 0.125, 0.25 and 0.5 mM. Fluorescence and absorbance were monitored for 30 minutes for HPK and HNE, respectively. Michaelis-Menten kinetics were established as well as the inhibitor constant, K_i, using the Morrison equation for tight binding inhibitors.[8]

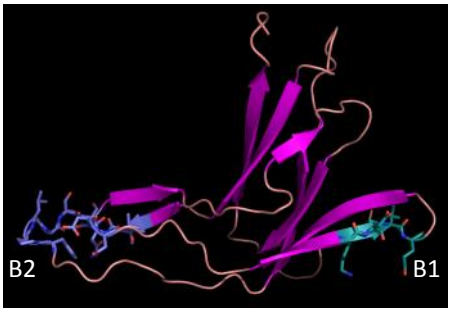

Figure 1: Predicted protein model structure of *F. necrophorum* ecotin highlighting the primary binding site (B1) and secondary binding site (B2).

Thrombin time (TT), prothrombin time (PT) and activated partial thromboplastin time (APTT) clotting assays were performed on human donor blood in duplicate on three separate days. Ecotin concentration ranged from 0 – 2 µM and incubation time with plasma was set at 120 seconds. Reagents and standard protocols were acquired from Diagnostic Reagents Ltd (Thame, Oxfordshire).

Results

The ecotin gene was present in 100% of the 26 strains tested. When translated, the protein sequence was found to be 159 amino acids long and highly conserved.

SWISS-MODEL and PyMol were used to create a protein model of *F. necrophorum* ecotin (Figure 1). The primary and secondary binding sites are predicted to be exposed on opposite sides of the protein.

Michaelis-Menten kinetics of HPK and HNE with their respective substrates were determined with no inhibitor present (Table 2).

Table 2: Values for maximal velocity (V_{max}) and substrate concentration required to reach half enzyme velocity (K_m) obtained using Michaelis-Menten non-linear regression analysis.

Enzyme	V_{max} (µmol min^{-1})	K_m (µM)
Plasma kallikrein	0.293	0.252
Neutrophil elastase	0.098	0.085

For each enzyme, the ratio of inhibited to uninhibited rate of reaction was plotted for the substrate concentration nearest the K_m (Figures 2 and 3). The Morrison equation was used to determine the estimated concentrations required to produce half maximal inhibition, known as the inhibition constant (K_i) (Table 3).

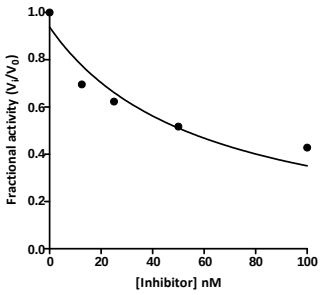

Figure 2: Activity of human plasma kallikrein equilibrated with ecotin at 0—100 nM, expressed as a ratio of the inhibited to uninhibited rate. Substrate concentration was 250 µM.

Table 3: K_i values calculated using the Morrison equation.

Enzyme	K_i value (µM)
Kallikrein	30.0
Neutrophil elastase	3.4

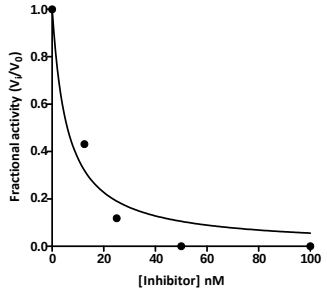

Figure 3: Activity of human neutrophil elastase equilibrated with ecotin at 0-100 nM, expressed as a ratio of the inhibited to uninhibited rate. Substrate concentration was 60 µM.

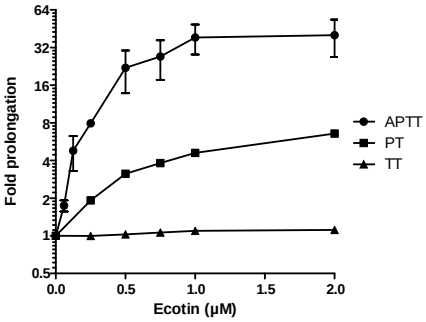

Figure 4: Prolongation of clotting time in normal human plasma upon incubation at 37°C for 120 seconds with ecotin at 0-2 µM. Mean normal clotting times were 26s for TT, 22s for PT and 65s for APTT.

At 2 µM, ecotin caused a 1.1-fold increase in clotting time for TT, 7-fold increase for PT and 40-fold increase for APTT (Figure 4).

Discussion

The K_i values in Table 3 demonstrate that ecotin inhibits human neutrophil elastase more potently than it does human plasma kallikrein (3.4 µM compared to 30 µM).

HNE is secreted by neutrophils and macrophages and has been shown to target virulence factors of pathogens such as *Shigella* and *Yersinia*.[9] Ecotin potently inhibited HNE, suggesting that one role may be to protect the organism from host proteases.

The inhibitory effect of ecotin on HPK suggests that ecotin may also play a role in regulating coagulation. PT assays measure efficiency of the extrinsic pathway, while APTT assays measure the intrinsic pathway.[10] At concentrations up to 2 µM, APTT was prolonged to a greater effect than PT, suggesting *F. necrophorum* ecotin has a greater inhibitory effect on the intrinsic pathway, by binding to enzymes such as kallikrein.

The K_i values suggest that the inhibition of HNE could be the primary function, in order to protect the bacteria. The inhibition of clotting mechanisms may be coincidental. The relevance of this inhibition during infections is yet to be understood.

References

1. Batty, A., Wren, M. W. D., *et al*. (2005) "Fusobacterium necrophorum as the cause of recurrent sore throat: comparison of isolates from persistent sore throat syndrome and Lemierre's disease." *Journal of Infection* 51(4): 299-306.
2. Kuppalli, K., Livorsi, D., *et al*. (2012) "Lemierre's syndrome due to Fusobacterium necrophorum." *The Lancet Infectious Diseases* 12(10): 808-815.
3. Tan, Z. L., Nagaraja, T. G. & Chengappa, M. M. (1996) *Fusobacterium necrophorum* infections: Virulence factors, pathogenic mechanism and control measures. *Veterinary Research Communications* 20, 113-140.
4. Smith, G. R., Wallace, L. M. & Noakes, D. E. (1990) Experimental observations on the pathogenesis of necrobacillosis. *Epidemiology and Infection* 104, 73-78.
5. Tadepalli, S., Stewart, G. C., Nagaraja, T. G. & Narayanan, S. K. (2008) Human *Fusobacterium necrophorum* strains have a leukotoxin gene and exhibit leukotoxic activity. *Journal of Medical Microbiology* 57, 225-231.
6. Seymour, J. L., Lindquist, R. N., *et al*. (1994) Ecotin Is a Potent Anticoagulant and Reversible Tight-Binding Inhibitor of Factor Xa. *Biochemistry* 33, 3949-3958.
7. Ulmer, J. S., Lindquist, R. N., *et al*. (1995) Ecotin is a potent inhibitor of the contact system proteases factor XIIa and plasma kallikrein. *FEBS Letters* 365, 159-163.
8. Morrison, J. F. (1969) Kinetics of the reversible inhibition of enzyme-catalysed reactions by tight-binding inhibitors, *Biochimica et Biophysica Acta (BBA) - Enzymology 185*, 269-286.
9. Weinrauch, Y., Drujan, D., *et al*. (2002) Neutrophil elastase targets virulence factors of enterobacteria. *Nature* 417, 91-94.
10. Bain, B. J., Bates, *et al*. (2011) *Dacie and Lewis Practical Haematology*. 11th ed. UK: Elsevier Health Sciences.