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Identification of Binding Site Residues of a Serine Protease Autotransporter

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Kellogg Honors College Capstone 2011

Abstract

Serine Protease Autotransporters of *Enterobacteriaceae* (SPATE) are multidomain proteins found in pathogenic enteric bacteria. They are characterized by a conserved serine protease function. These proteins are comparatively large (>100 kDA) and are comprised of a signal sequence, a passenger domain, and a translocater domain. The passenger domain is responsible for the protein's virulence activity. Temperature sensitive hemagglutinin (Tsh) is used as a model SPATE in our studies. In the present study we aim to define the substrate binding site of Tsh. Sequence alignment was performed using Tsh and another autotransporter called Hap, for which the potential substrate binding site has been published. From this we identified five potential residues: G254, L279, T280, A281, and N290. Based on our hypothesis, mutations introduced at these five residues are expected to deform the substrate groove and thereby inhibit the correct positioning of the substrate at the active site. This would result in decreased proteolytic function. Therefore, to test this hypothesis we introduced single point mutations at these five residues using PCR based site directed mutagenesis. SDS-PAGE and western blotting were then used to analyze the secretion pattern of the mutants. A functional analysis was performed using a Tsh-specific oligopeptide assay and a mucin assay. The secretion analysis showed that the mutations did not impact the proper secretion of the mutants. The results of the cleavage assays showed a significant reduction in the proteolytic function of the mutants. These results show that the five residues that we have identified are crucial for the proteolytic activity of Tsh, which suggests a role of these residues in forming the Tsh substrate binding site

Introduction

Gram-negative bacteria have an outer membrane encompassing their cell walls which hinders the secretion and uptake of substances such as proteins. As a result, gram-negative bacteria have developed systems to aid in the secretion of proteins to the extracellular space. One of the protein secretion pathways is called the Autotransporter (AT) pathway. This is a two-step pathway that transports virulence factors to the extracellular environment. These virulence factors contribute greatly to bacterial pathogenicity. The autotransporter proteins secreted via this pathway are fairly large (>100 kDA). These proteins are comprised of three domains: a N-terminal signal sequence, a passenger domain, and a translocater domain. The N-terminal signal sequence is recognized by the Sec translocon allowing the protein to translocate across the inner membrane. The passenger domain folds into a functional product upon secretion and is responsible for virulence activity, and lastly the C-terminal translocator domain directs translocation across the outer membrane.

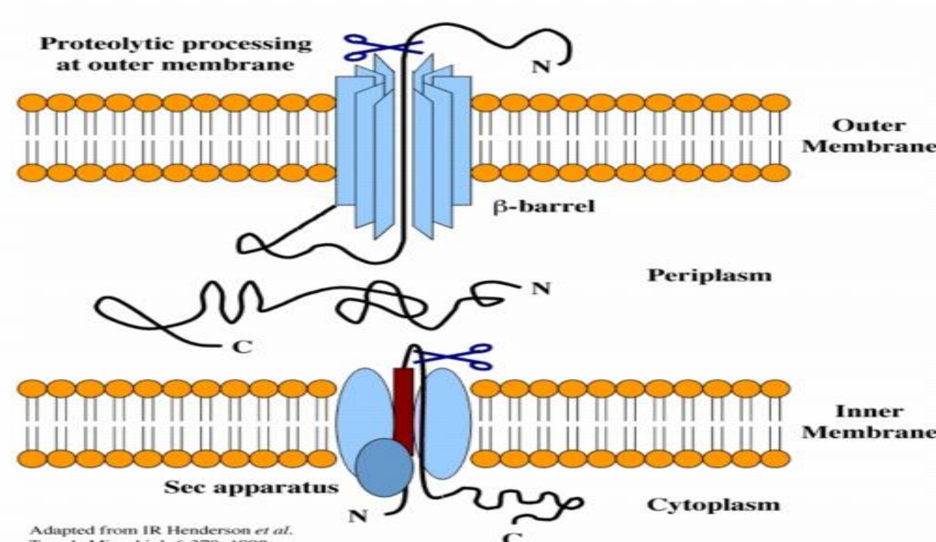


FIG. 1. Mechanism of autotransporter secretion. The N-terminal signal sequence directs the AT to the Sec apparatus and facilitates translocation across the inner membrane. Upon entering the periplasm, the signal sequence is cleaved, while the C-terminus beta domain inserts itself into the outer membrane forming a beta barrel that aids in translocation of the passenger domain across the outer membrane, which then folds into a functional protein.

The research in our lab focuses on a particular family of AT called Serine Protease ATs of the Enterobacteriaceae (SPATEs), which is characterized by their virulence roles. SPATEs possess conserved motifs in their passenger domains (GDSGS) that contribute to their serine proteolytic function. More than 20 members of the SPATE family have been identified in pathogenic enteric bacteria. The model SPATE used in our lab is temperature sensitive hemagglutinin (Tsh). It is secreted by avian pathogenic and human uropathogenic *E. coli*, and functions as an adhesin and a protease.

Table 1. Examples of serine protease autotranspoters of the enterobacteriaceae

Protein	Organism	Disease	Function
EspP	EHEC	Bloody diarrhea; hemorrhagic colitis	Cytotoxin
EspC	EPEC	Diarrhea	Enterotoxin
EspI	STEC	Diarrhea, renal failure	Unknown
EpeA	EHEC	Bloody diarrhea; hemorrhagic colitis	Unknown
EatA	ETEC	Diarrhea	Cytopathic effects on intestinal cells
Hbp	*Escherichia coli*	Wound infections and septicemia	Sequestering of heme
Tsh	APEC	Collibacillosis, septicemia, and airsacculitis	Adhesin, protease, Hemagglutinin
SepA	*Shigella felxneri*	Shigellosis	Inflammation in intestine, tissue invasion
Pic	EAEC; *Shigella flexneri*	Shigellosis, bloody diarrhea	Cytopathic effects on intestinal cells, mucinase, hemagglutinin

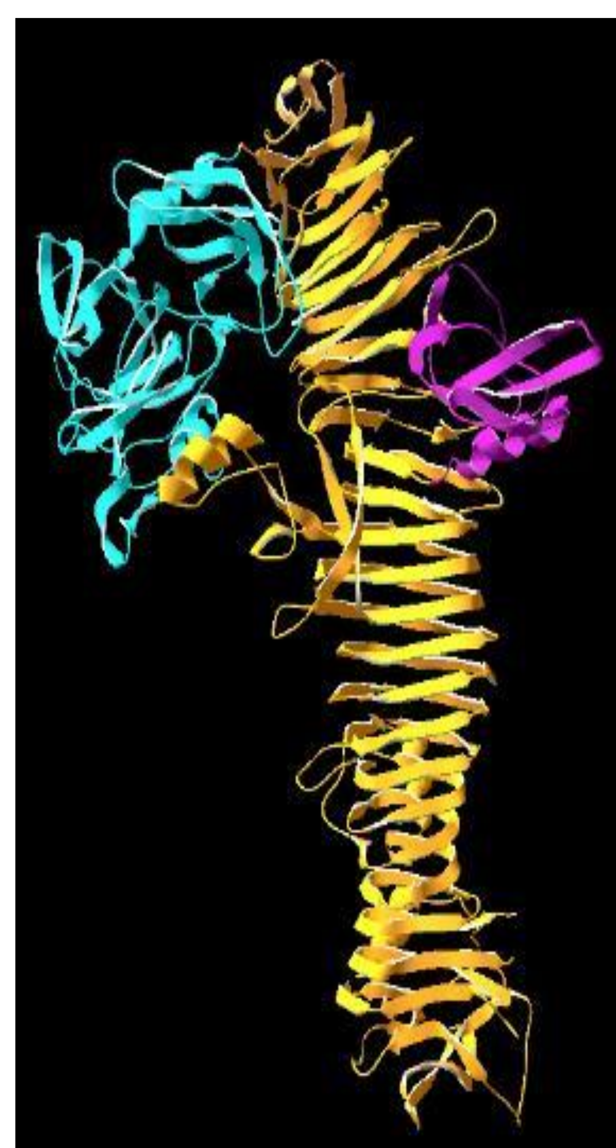


FIG. 2. Crystal structure of the passenger domain of Hpb (PDB ID 1wxr)-a Tsh Homolog

Research Overview

The goal of our research is to determine the amino acid residues making up the substrate binding site of Tsh. In order to determine the potential binding site residues, the crystal structures of the serine protease alpha-chymotrypsin covalently bound to a tosylate inhibitor and the serine protease domain of Hbp were superimposed in Swiss PDB Viewer. The coordinates of the tosylate inhibitor in the superimposed conformation were then merged with the Hbp serine protease domain coordinates, enabling us to search for residues around the inhibitor. Sequence alignment was also performed between Tsh and Hap, another autotransporter for which the potential substrate binding site has been identified. Using these tools, we were able to identify the following residues as potential residues of the Tsh substrate groove: G254, L279, T280, A281, and N290. Based on our hypothesis, mutations introduced at these five residues are expected to deform the substrate groove and thereby inhibit the correct positioning of the substrate at the active site. This would result in decreased proteolytic function.

To test these hypotheses, PCR based site-directed mutagenesis will be used to mutate the binding site residues of Tsh. After mutation, the residues will be subjected to a secretion profile analysis using SDS-PAGE and western blotting to confirm secretion of Tsh. Cleavage assays such as the Tsh-specific oligopeptide assay and mucin assay will then be used to study the proteolytic function of the mutants.

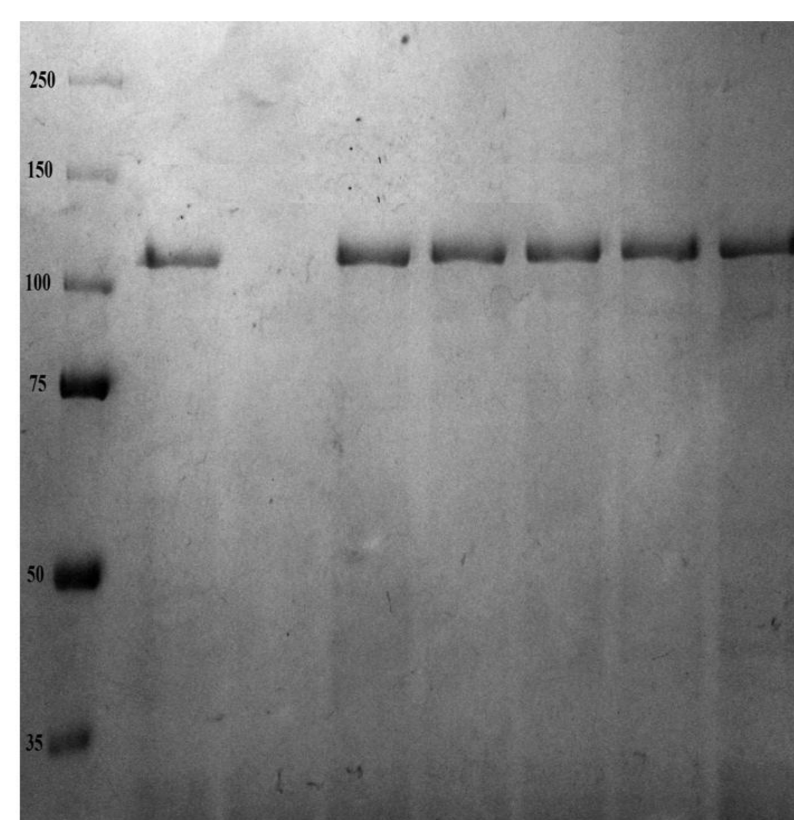
Mutagenesis

1. Primers for the mutant were constructed.
2. PCR was used to amplify the mutant DNA.
3. Mutated plasmid was used to transform bacteria (*E. coli* K-12 strain)
4. Extraction of plasmid to verify sequence using Qiagen miniprep kit.
5. DNA sequencing was done at City of Hope

Results

Secretion Profile Analysis

A Marker WT CV G254A L279R T280A A281R N290A



B

FIG. 3. Secretion profiles of Tsh mutants. The samples are resolved by SDS-PAGE (10% polyacrylamide) and silver stained. The samples are also transferred to a nitrocellulose membrane for immunoblot analysis. Wild type (wt) and S259A serve as positive controls while XL1-Blue strain with a cloning vector serves as a negative control (Cv). A: silver stained SDS-PAGE gel; B: western blot.

Oligopeptide Cleavage Assay

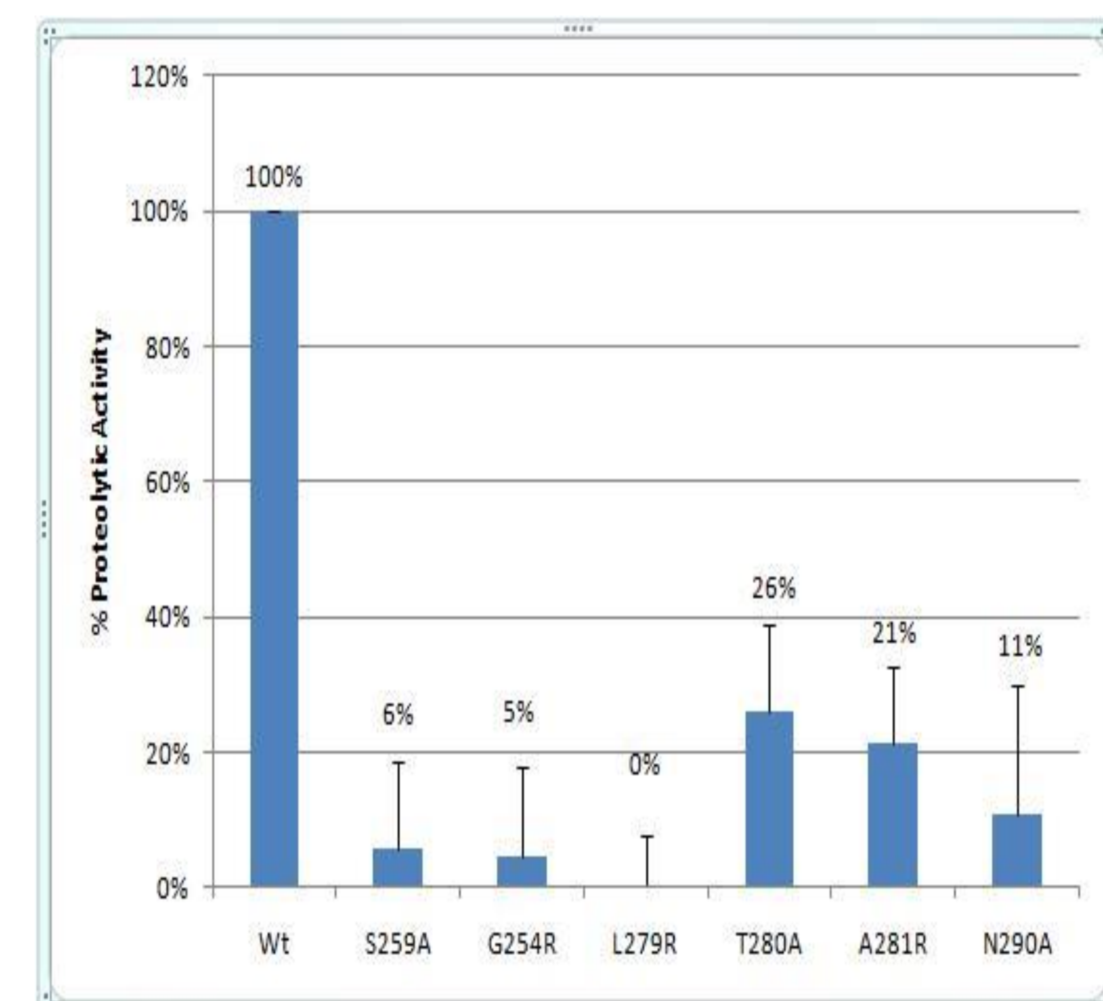


FIG. 4. Oligopeptide cleavage assay results of the three non-conserved mutants. Concentrated supernatants were incubated at 37°C with 1mM p-NA conjugated oligopeptide in MOPS buffer at pH 7.3 for 15 hours. S259A serves a negative control for proteolytic activity. The substrate cleavage of each mutant was normalized based on the level of S259A before comparing their activity with wildtype. The reaction were performed in triplicates and the results were averaged. The interaction between the conserved and non-conserved constricts is shown.

Mucin Assay

Wildtype	+++
S259A	-
G254R	-
L279R	-
T280A	++
A281R	++
N290A	+

Table 2. Proteolytic activity of a conserved and non-conserved mutant on a porcine gastric mucin gel. Culture supernatant of wildtype and each mutant are collected and concentrated using a 10-K centrifuge filter. 1mM PMSF is used as a serine protease inhibitor. The samples are resolved by SDS-PAGE (10% polyacrylamide) and stained with Schiffs reagent. Wild type (wt) serves as a positive control and S259A serves as a negative control.

+ signifies intensity of cleavage

- signifies absence of cleavage

Conclusions

This results show that mutations at the five residues, G254, L279, T280, A281, and N290 lead to extremely low proteolysis of the Tsh specific substrate, indicating that these residues are crucial for the protein's proteolytic function. This supports our hypothesis and strongly suggests a role of these residues in forming the substrate binding site of Tsh.

Methods

Molecular modeling: Graphics were generated using the coordinates of the published crystal structure of Hbp (Otto, Sijbrandi et al. 2005); PDB ID: 1WRX) and the VMD program (Humphrey, Dalke et al. 1996) version 1.8.6.

Site-directed Mutagenesis. Site-directed mutagenesis of plasmid pYA3418 carrying *tsh* was performed according to the QuickChange protocol (Stratagene). Sequencing of all constructs was performed at the DNA sequencing lab at City of Hope.

Culture Preparation and Induction. *E. coli* XL1 Blue cells were grown at 37□C in Luria broth medium containing 100 ug/ml ampicillin An optical density of 0.6 reached at 600nm, after which further expression of Tsh was induced by the addition of IPTG for 3 hours. Supernatants were then collected by centrifugation. The supernatants were concentrated (80X) by filter centrifugation using 100-K filters at 5000 rpm. The concentration of the samples was analyzed using the Bradford assay.

SDS-PAGE Gel Electrophoresis and Silver Staining. 8% polyacrylamide gels were prepared. The samples were run on gels and visualized using silver staining.

Immunoblot. Proteins from the gels were transferred to the nitrocellulose membranes which were then blocked overnight with 5% nonfat milk in TTBS. The membrane was incubated with rabbit anti-Tsh ab (1:5,000) was for 2h followed by an incubation with horseradish peroxidase-conjugated anti-rabbit IgG (1:20,000) for 2h. Development of the blots was performed using chemiluminiscence (Pierce).

Oligopeptide Cleavage Assay. Proteins were incubated with 1mM p-NA conjugated oligopeptides in MOPS buffer at pH 7.3 in 96-well plates (triplicates of each). The plates were incubated for 15 hrs at 37°C and the absorbance readings were measured at 405nm.

Mucin Assay. Proteins were run on 8% polyacrylamide gels containing porcine mucin. The gels were incubated in mucinase buffer at 37°C for 40 hrs followed by incubation with 25% isopropanol and 10% acetic acid 2 hrs and 10% isopropanol and 10% acetic acid for 12 hrs. It was then incubated with 10% acetic acid for 10 hrs and 1% periodic acid and 3% acetic acid for 1 hr. The gel was rinsed with ddwater, stained with Schiffs reagent and bleached with 0.5% sodium metabisulfite.

Acknowledgements

The funding for this project was provided by the Department of Education's College Cost Reduction and Access Act (CCRAA).

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