CHARACTERIZATION OF ANTI-TCDB DARPIN DISULFIDE MUTANTS

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Characterization of Anti-TcdB DARPin Disulfide Mutants

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Clostridioides difficile (*C. difficile*), the microbe responsible for *Clostridioides difficile* infection (CDI), is a common nosocomial infection that exerts its pathogenicity primarily by two toxins, TcdA and TcdB. Designed ankyrin repeat proteins (DARPins) are an emerging approach of protein therapeutics to combat disease beyond the limitations of antibiotics and monoclonal antibodies. Disulfide bonds are commonly used in other facets of protein engineering to enhance stability, but their use has not been well documented with DARPins. Previously, the Chen lab engineered DARPins that have been demonstrated to be effective at neutralizing TcdB. However, these DARPins are not protease-stable, which is a barrier for effective delivery in downstream therapeutic contexts. To address this, disulfide bonds were introduced in order to increase the stability of the DARPins and thus increase resistance to protease digestion. The subsequent structure, stability, and neutralization activity are assessed to ascertain the effects of bolstering tertiary structure. The formation of disulfide bonds is confirmed by the comparison of mutants in oxidizing and reducing conditions. Elapsed trials with trypsin and chymotrypsin incubation demonstrate protease stability. Overall, the addition of disulfide bonds is demonstrated to

improve stability at minimal cost to neutralization activity. The successful characterization of these disulfide mutants may grant continuing insight into future protein engineering applications and aid the development of a therapeutic anti-TcdB DARPin.

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NOMENCLATURE

BCA	Bicinchoninic acid
BME	2-Mercaptoethanol
BSA	Bovine serum albumin
C. difficile	*Clostridioides difficile*
CDI	*Clostridioides difficile* infection
DARPin	Designed ankyrin repeat protein
E. coli	*Escherichia coli*
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria-Bertani
OD600	Optical density measured at 600 nm
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline buffer
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

1. INTRODUCTION

Designed ankyrin repeat proteins (DARPins) are an emerging approach of protein therapeutics to combat disease beyond the limitations of antibiotics and monoclonal antibodies (mAbs). The small molecular weight of this novel class of proteins gives it an advantageous pharmacokinetic profile, in addition to its high variability and high specificity-binding capacity, heat stability, and easy expression[1]. Furthermore, DARPins possess key qualities associated with low immunogenicity, having high stability, no aggregation tendency, and lacking an Fc domain[2]. Subsequently, the therapeutic potential of DARPins is great and is expanded by the multi-specificity and functional modifications that can be engineered.

Major potential for DARPins lies in the treatment of *Clostridioides difficile* (*C. difficile*), an obligate anaerobe, Gram-positive, spore-forming bacterium that is the leading cause of nosocomial infectious diarrhea worldwide[3]. Found in the gastrointestinal tract, *C. difficile* exerts its virulence by toxin A (TcdA) and toxin B (TcdB) and causes *C. difficile* infections (CDI)[4]. Standard treatment of CDI currently comprises the use of several antibiotics including vancomycin, metronidazole, and fidaxomicin[3]. The Centers for Disease Control and Prevention (CDC) have *C. difficile* marked as an urgent threat in regard to antibiotic resistance, underscoring the need for innovative therapeutics to lower the 12,800 deaths that occur from CDIs each year[5].

There are no DARPins currently in clinical use, although several candidates have progressed to clinical trials[6,7]. Previously, the Chen lab engineered DARPin constructs that were shown to be effective at neutralizing TcdB[8]. Additional DARPin constructs engineered were not protease-stable, which is a barrier for combating *C. difficile* activity in the small intestine[4]. Given the residence of *C. difficile* in the bowels, oral administration of therapeutics will allow for

increased bioavailability relative to systemic administration for this neutralizing mechanism of action. Orally administered DARPins must therefore be able to withstand the digestive processes all proteins are subjected to. To address this, disulfide bonds were introduced in order to increase the rigidity and stability of the DARPins and thus engineer them to be resistant to protease digestion. Three disulfide mutants were engineered – 1ss24, 41ss73, and 139ss148 – from parent DARPin T10-2 (Figure 1.1). Naming accordingly reflects the location of mutated cysteine residues, and thus putative disulfide formation, at the 1st and 24th residue, 41st and 73rd residue, and 139th and 148th residue accordingly.

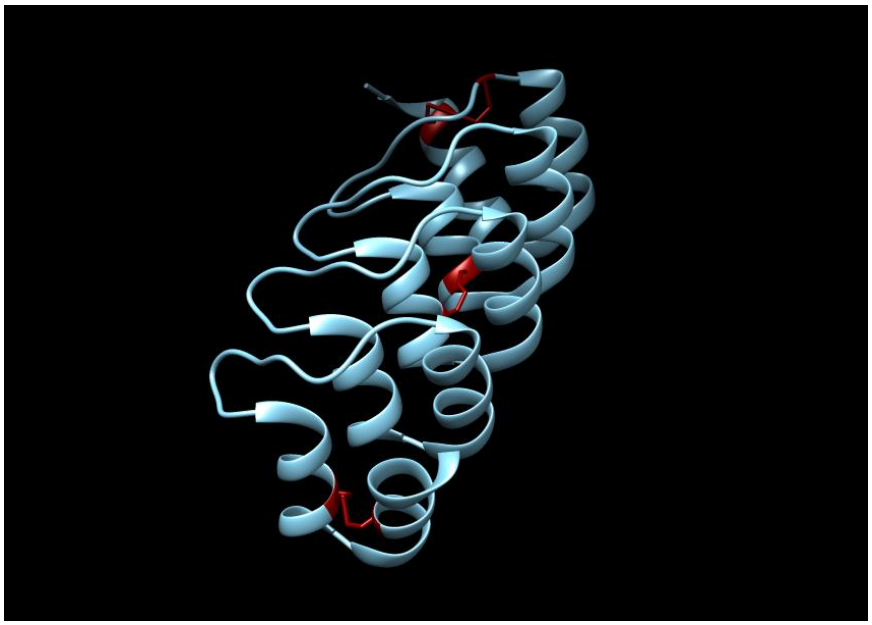

Figure 1.1: Locations of disulfide bonds are highlighted in red on a model of T10-2 from a Chimera visualization[9]

Enhanced protein stability is a desirable goal for many biomedical applications, expanding the range over which a protein can retain its conformation and function. Disulfide bonds contribute to this stability by providing conformational constraints, thereby providing support to withstand a variety of environmental stressors[10] and conferring protease resistance[11]. Although disulfides have been reported to add 2.5-5.1 kcal/mol to the thermodynamic stability of

a protein[12], disulfides that destabilize structure have also been reported. The determinants for if inserted disulfide bonds will be stabilizing or destabilizing are under study[13], and various bioinformatic models have been developed to better elucidate these elements and predict protein stability[14].

The formation of disulfide bonds is primarily influenced by the physical distance between cysteine residues, surrounding pH, and redox environment[15]. During protein synthesis, the cellular environment maintains reduced cysteine thiols; upon exiting the cell, where disulfide proteins most often enact their functions, exposure to oxygen provides an oxidizing environment in which these bridges can subsequently form[13]. Similarly, protein release upon cell lysis exposes DARPins to an oxidizing environment. DARPins are fast-folding proteins that assemble based on short-range interactions, favoring successful disulfide formation compared to slower folding proteins[16,17].

Disulfide bonds added in regions of flexibility that create large loops have been reported to increase stability, largely due to the ability of the backbone to adjust to optimal geometry for the disulfide bond[18]. In this study, residues linking ankyrin repeats within 2.3 Å were identified and mutated into cysteine residues. While the peptide backbone is planar and limited in rotation, the C_α–C and N–C_α bonds are able to rotate[19], meaning the primary consideration for disulfides bridges here was ensuring the physical proximity of cysteine β-carbons and avoiding introducing steric strain in the native conformation.

Despite its growing utility in other facets of protein engineering[13], the use of disulfide bonds has not been well documented with DARPins. The enhancement in stability that disulfide bridges can provide offers a promising avenue to improve the activity of neutralizing DARPins. The characterization of these DARPin mutants is subsequently necessary to determine structure,

stability, binding affinity, and neutralization activity. An increase in protease-stability at marginal cost to neutralization activity will be informative to future studies on protein engineering in developing a clinically effective DARPin against TcdB.

2. METHODS

2.1 Disulfide Mutant Expression and Purification

Shuffle *E. coli* containing plasmid DNA encoding the disulfide mutants were inoculated in 5 mL of LB broth with 50 µg/mL Kanamycin and cultured at 37ºC. After 16 hours, each culture was transferred into 100 mL of LB broth with 50 µg/mL Kanamycin and cultured until OD600 values between 0.4 and 0.8 were measured with a spectrophotometer. Protein expression was induced with 0.4 mM IPTG and kept at 18ºC for 18 hours. Cells were collected by centrifugation and lysed via sonification. Samples were centrifuged and the supernatant collected for purification of soluble proteins. Purification was performed by Ni-NTA affinity column chromatography. Two rounds of washes were performed twice: one set with PBS and the other with PBS containing 30 mM imidazole. Purified protein was eluted with PBS containing 150 mM imidazole. The DARPins were buffer exchanged into PBS and concentrated by 10k Amicon ultra centrifugal filters. SDS-PAGE was performed to confirm expression and purification. Samples were flash-frozen with liquid nitrogen and kept at -80 ºC.

2.2 Disulfide Mutant Concentration Determination

To ascertain protein concentrations from measured nanodrop concentrations, a BCA protein assay was used to quantify total protein and determine a corresponding extinction coefficient. A standard curve from 0 to 2000 µg/mL was created using BSA.

2.3 Disulfide Bond Formation Confirmation

Disulfide bond confirmation was performed by analysis of varied SDS-PAGE run conditions. Two samples of each DARPin were electrophoresed: one sample with loading buffer containing BME, and one without. Samples were heated at 95ºC for five minutes, then loaded onto a 12% acrylamide SDS-PAGE at 200V for 45 minutes. Staining was performed with Coomassie blue.

2.4 Protease Digestion Trials

Disulfide DARPin stability was assessed in the presence of trypsin and chymotrypsin. DARPins were diluted to 0.5 mg/mL in PBS. 100 µL of trypsin slurry was combined with each DARPin and incubated for 3 hours. Similarly, 25 µL of chymotrypsin slurry was combined with each DARPin and incubated for 3 hours. At each hour (0, 1, 2, 3), samples were collected and kept at -20ºC. A native PAGE was run to assess protein conformation and stability. The parent DARPin T10-2 was used as a control.

2.5 Cell Viability Assay

The ability of DARPins to maintain cell viability in the presence of TcdB was measured with Vero E6 cells. Cells were seeded at a density of 1.5×10^3 cells/well overnight. DARPins were incubated with either PBS, 1 mg/mL trypsin, or 0.5 mg/mL chymotrypsin for 1 hour at 37ºC. For each DARPin, a five-fold serial dilution series was constructed from 0.0016 nM to 125 nM. These protein dilutions were added to the cells following incubation, as was 1.1 pg/mL TcdB. Cells were thereafter incubated at 37ºC for 72 hours. Cell viability was determined from ATP levels by measuring luminescence with CellTiter-Glo. All aforementioned procedures were

repeated with T10-2 and 139ss148 altering the initial incubation to be with PBS, 0.5 mg/mL chymotrypsin, or 1 mg/mL chymotrypsin.

3. RESULTS

3.1 Disulfide Bond Confirmation

As the proteins were denatured by the presence of SDS and application of heat, differences in band migration can be attributed to the presence of disulfide bonds. The presence of a disulfide bond alters the migration of the protein such that DARPins that exhibit the same run pattern in reducing conditions (with BME) and oxidizing conditions (without BME) lack a disulfide bond; conversely, DARPins with dissimilar run patterns in these conditions contain a disulfide bond. As expected, T10-2, which lacks cysteines, displayed the same migration pattern in reducing and oxidizing conditions (Figure 3.1). Similarly, 41ss73 displayed the same run pattern in both conditions and was determined to not have formed a disulfide bond. The 1ss24 and 139ss148 mutants displayed a difference in band migration between redox conditions and were confirmed as having successfully formed disulfide bonds. Given the naturally oxidizing conditions of the exposed in vitro setting and close proximity, the absence of a disulfide bridge in 41ss73 may be the result of conformational strain imposed on the backbone to accommodate such a crosslink.

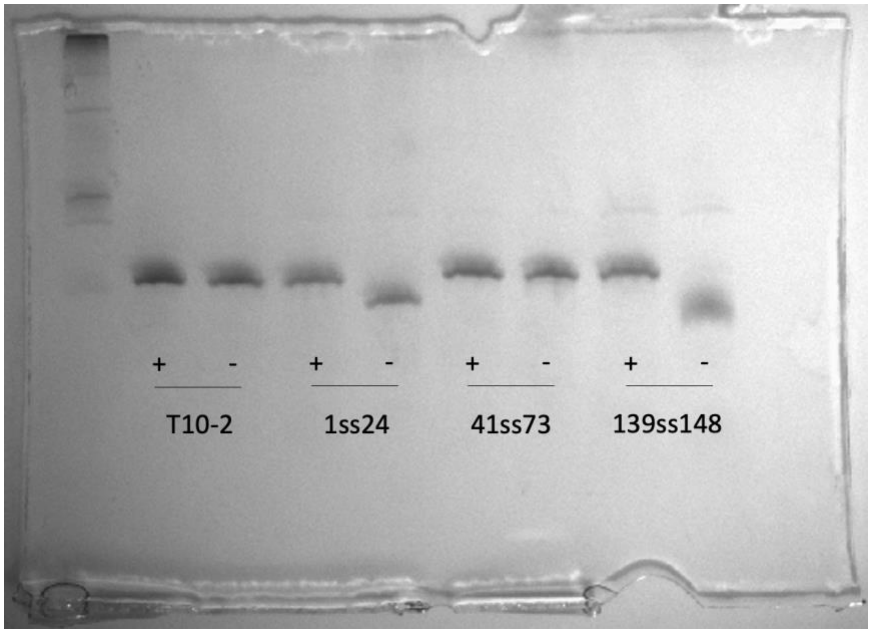

Figure 3.1: SDS-PAGE of T10-2 and disulfide mutants in reducing and oxidizing conditions. + indicates the addition of BME, - indicates the absence of BME.

3.2 Disulfide Mutants are Stable in the Presence of Trypsin

Disulfide DARPin mutants were incubated with trypsin for three hours to assess if original conformation was retained in the presence of proteases found in the intestinal tract. When electrophoresed on a native PAGE, all DARPins exhibited a consistent appearance over three hours (Figure 3.2, 3.3). This confirms the trypsin stable nature of the disulfide mutants. The introduction of the disulfide bonds did not interfere with the trypsin resistant nature of T10-2 from which the mutants were created.

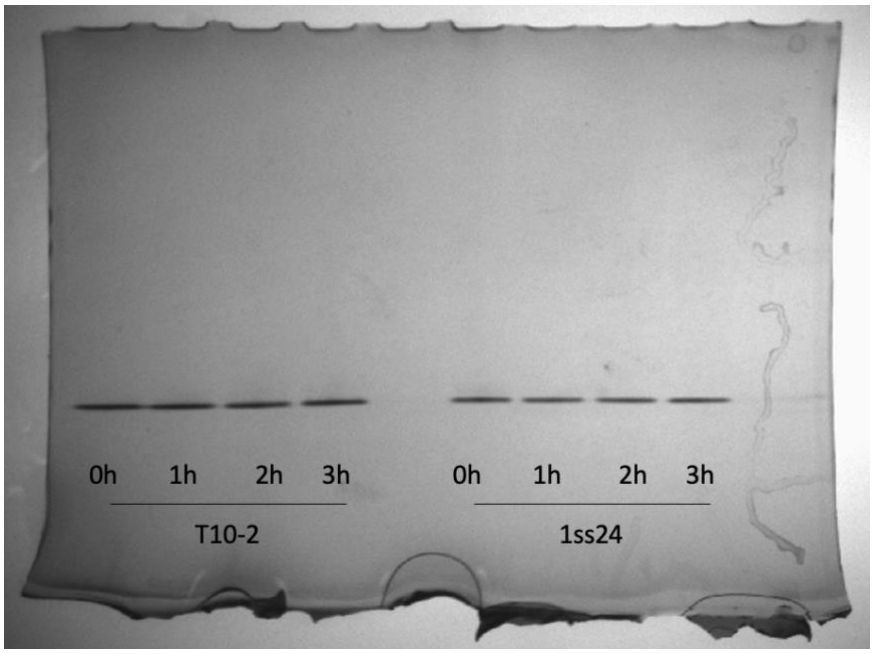

Figure 3.2: Native PAGE of T10-2 and 1ss24 after incubation with trypsin over 3 hours.

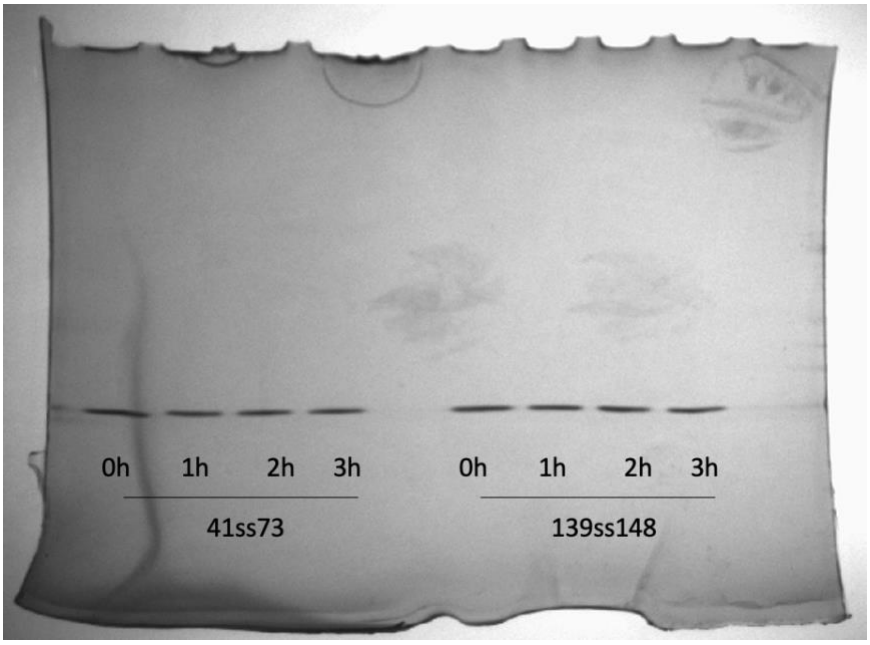

Figure 3.3: Native PAGE of 41ss73 and 139ss148 after incubation with trypsin over 3 hours.

3.3 Disulfide Mutants are Stable in the Presence of Chymotrypsin

With trypsin stability established, disulfide DARPin mutants were then incubated with chymotrypsin for three hours to assess if they likewise retained their original conformation despite protease activity. From native PAGE analysis, the initial DARPin conformation is revealed to be best maintained over the course of 3 hours in 1ss24 and 139ss148 (Figure 3.4, 3.5). In contrast, parent T10-2 is digested by chymotrypsin; the progressively dimmed protein band intensity over time in comparison and smear seen in hours 1-3 indicates the loss of conformation. Sustained band intensity by disulfide mutants in the presence of trypsin and chymotrypsin indicate protease resistance.

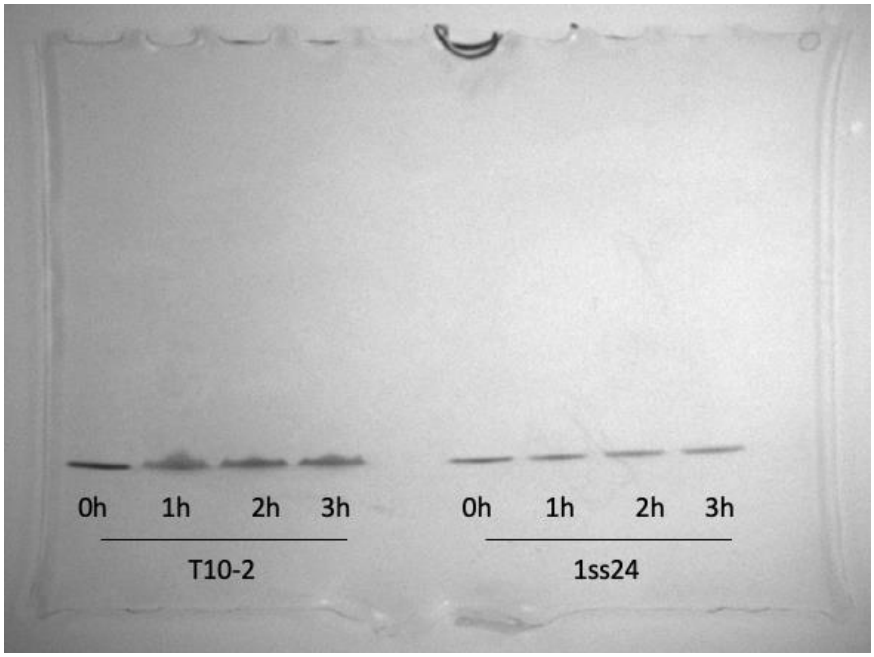

Figure 3.4: Native PAGE of T10-2 and 1ss24 after incubation with chymotrypsin over 3 hours.

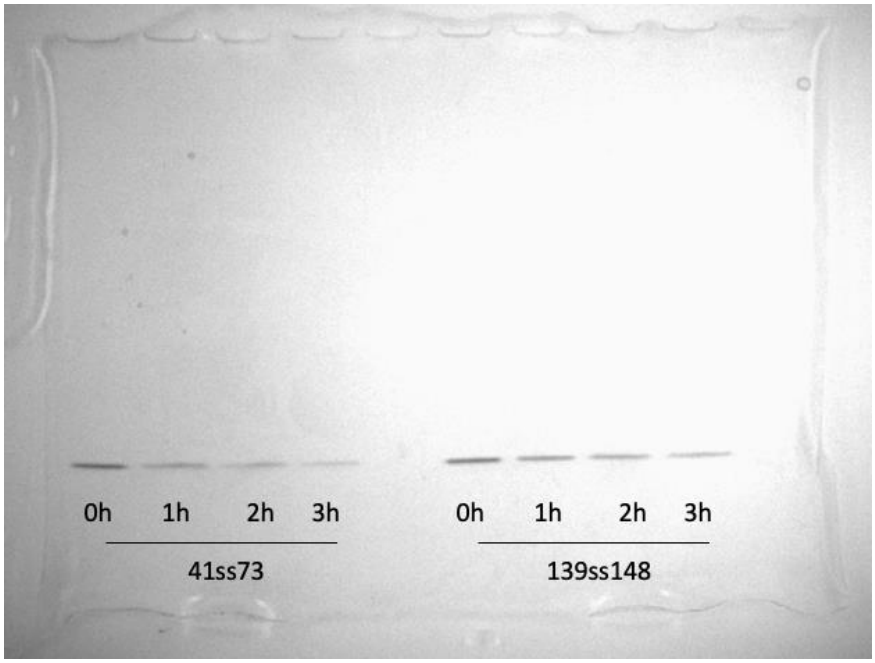

Figure 3.5: Native PAGE of 41ss73 and 139ss138 after incubation with chymotrypsin over 3 hours.

3.4 DARPin Disulfide Mutant 139ss148 and T10-2 Display Comparable Neutralization Activity

The effect of DARPin concentration when comparing cell viability in the presence and absence of proteases models physiological conditions that DARPins would be administered in for downstream therapeutic applications. Consistent with lack of disulfide bond formation and poorer performance in the digestion trials, 41ss73 demonstrated little neutralization activity in the cell viability assay in the presence of chymotrypsin (Figure 3.6).

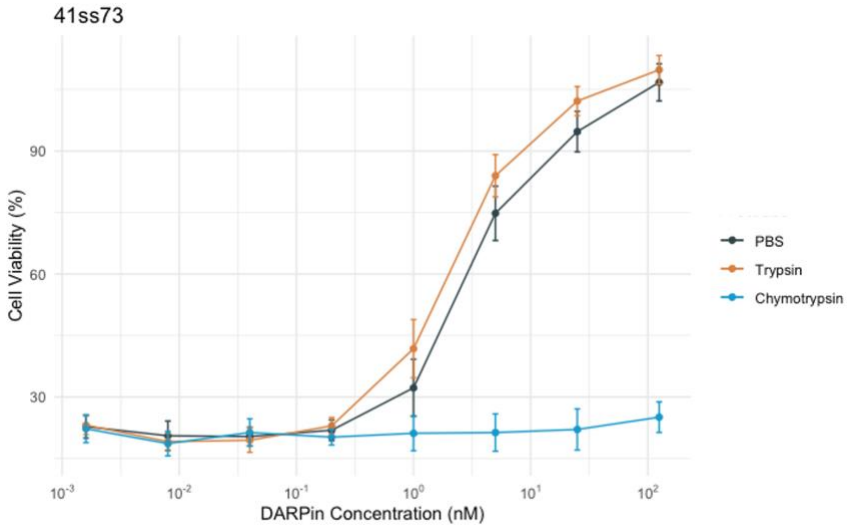

Figure 3.6: Cell rescuing activity of 41ss73 against TcdB in the presence of trypsin and chymotrypsin

1ss24 displayed robust neutralization activity (Figure 3.7), and 139ss148 had the best activity of the disulfide mutants in the presence of chymotrypsin (Figure 3.8). The enhanced performance seen in these two mutants relative to 41ss73 reflects the protease resistance observed in the protease digestion trials.

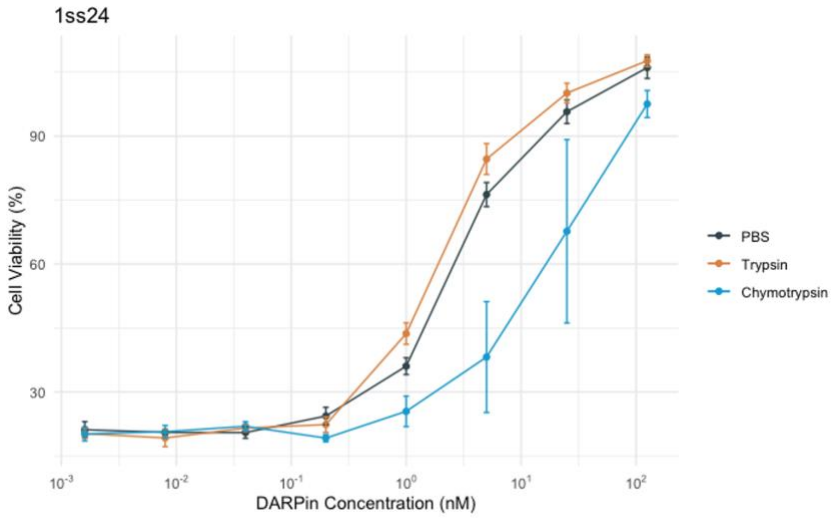

Figure 3.7: Cell rescuing activity of 1ss24 against TcdB in the presence of trypsin and chymotrypsin

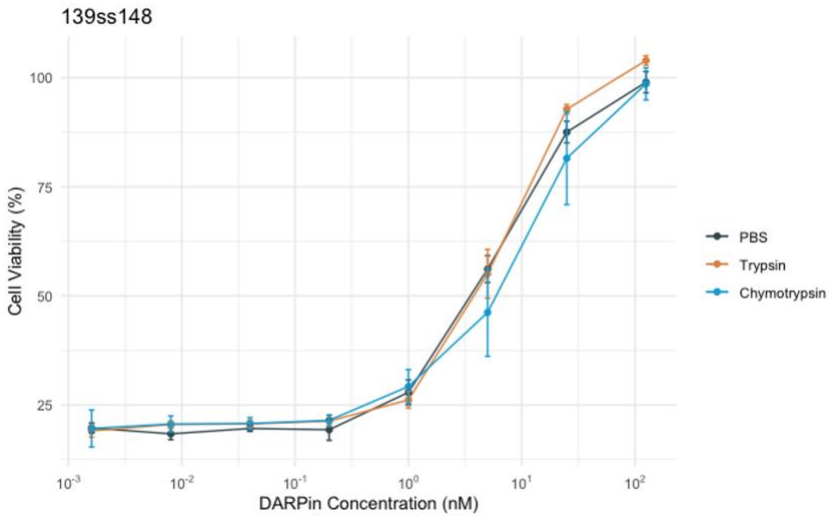

Figure 3.8: Cell rescuing activity of 139ss148 against TcdB in the presence of trypsin and chymotrypsin

Given the robust performance of 139ss148, its activity was assessed in varied chymotrypsin concentrations against T10-2 from which it was derived. Between 0.5 mg/mL and 1.0 mg/mL of chymotrypsin, 139ss148 was similarly effective in rescuing cells from the cytotoxic effects of TcdB (Figure 3.9). T10-2 also exhibited consistent rescuing activity (Figure 3.10). Overall, the performance of the two DARPins was comparable, with both 139ss148 and T10-2 exhibiting similar neutralization profiles.

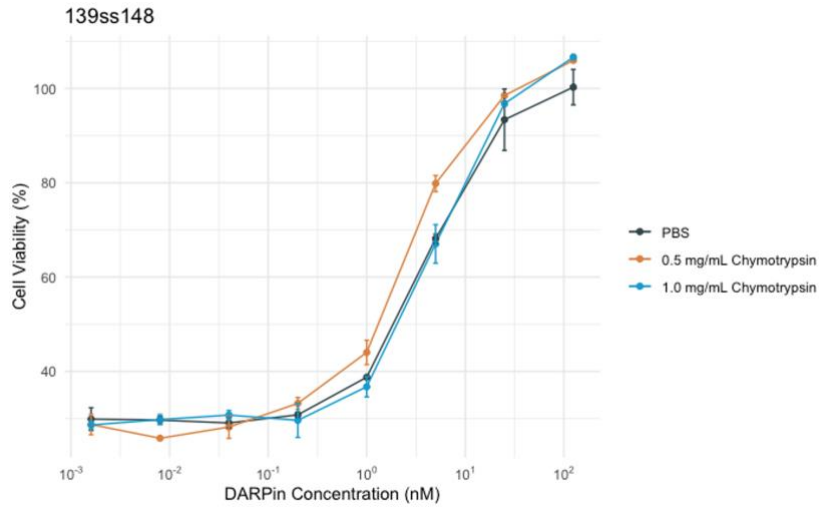

Figure 3.9: Cell rescuing activity of 139ss148 against TcdB in the presence of varied chymotrypsin concentrations

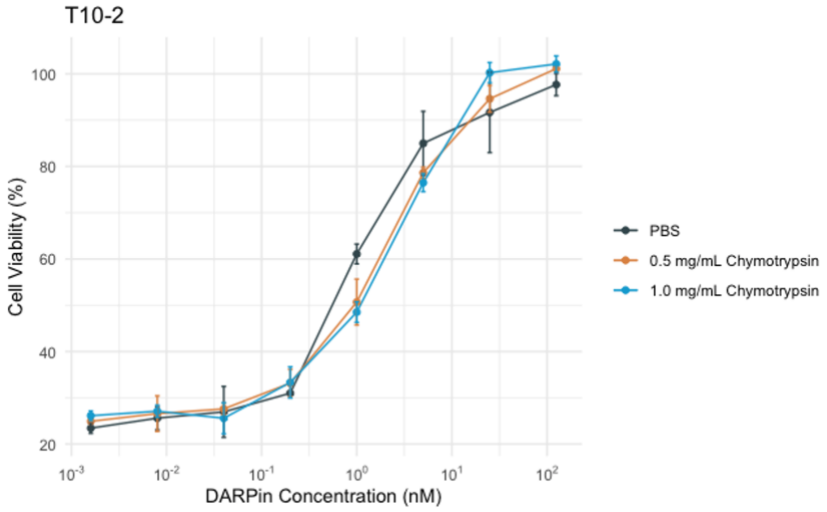

Figure 3.10: Cell rescuing activity of T10-2 against TcdB in the presence of varied chymotrypsin concentrations

4. CONCLUSION

4.1 Disulfide Bond Effects

Select disulfide mutant DARPins did appear to have enhanced stability, although not subsequently demonstrating improved neutralization activity. The introduction of disulfide bonds had varied augments on protease stability. 1ss24 and 139ss148 chymotrypsin resistance was superior to that of 41ss73, suggesting that the presence of a disulfide bond at the N or C terminus is more critical to maintaining protein conformation than a bond in the central ankyrin domains. As protein unfolding is required before cleavage can occur, the maintenance of secondary and tertiary structures subverts this process. By introducing these disulfide bridges at the terminals of the DARPin, access to cleavage sites is limited and allows for conformational stability despite protease activity.

4.2 Future Directions

DARPins are generally resistant to mutation and stable in the established repeated ankyrin structures. Here, the introduction of disulfides and stability was experimentally verified. 139ss148 was demonstrated to have similar performance to T10-2 from which it was created. Ultimately, a DARPin with improved performance to that of T10-2 is desirable, and various avenues exist to engineer such a protein. Among these, the combination of the disulfide bond at the 1st and 24th residues with the bond at 139th and 148th residues may exhibit further improved stability and neutralization activity. This introduces the challenge of ensuring optimal disulfide bond formation, as the presence of more than two cysteine residues expands the possible combinations of disulfide bond formation[20–22]. However, proper formation is still enhanced in

small, fast-folding proteins, and promoted through the use of proteins such as protein disulfide isomerase[23]. As demonstrated here, singular disulfide bonds were limited in their capacity to enhance TcdB neutralization. Although these alone were insufficient for improved activity, additional studies in tandem with other stabilizing modifications may prove useful to develop a better anti-TcdB DARPin that can be used to help combat *C. difficile*.

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