Protease-resistant single-domain antibodies inhibit Campylobacter jejuni motility

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Camelid heavy-chain antibody variable domains (VHs) are emerging as potential antimicrobial reagents. We have engineered a previously isolated VH (FlagV1M), which binds Campylobacter jejuni flagella, for greater thermal and proteolytic stability. Mutants of FlagV1M were obtained from an error-prone polymerase chain reaction library that was panned in the presence of gastrointestinal (GI) proteases. Additional FlagV1M mutants were obtained through disulfide-bond engineering. Each approach produced VHs with enhanced thermal stability and protease resistance. When the beneficial mutations from both approaches were combined, a hyperstabilized VH was created with superior stability. The hyperstabilized VH bound C. jejuni flagella with wild-type affinity and was capable of potently inhibiting C. jejuni motility in assays performed after sequential digestion with three major GI proteases, demonstrating the remarkable stability imparted to the VH by combining our engineering approaches.

Keywords: C. jejuni/motility/protease resistance/protein stability/VH

Introduction

Campylobacter jejuni is a non-spore forming gram-negative bacterium and one of the most common food-borne pathogens in humans (Ma, 2011). Infected individuals suffer from symptoms ranging from acute diarrhea to severe neurological disorders such as Guillain–Barré syndrome, reactive arthritis and inflammatory bowel disease (Altekruse et al., 1999; Hughes et al., 1999; Willison and O’Hanlon, 1999; Humphrey et al., 2007; Ma, 2011). Strategies to control or eliminate Campylobacter prevalence in poultry, particularly in chickens, are expected to improve poultry product safety and reduce the incidence of Campylobacter-induced gastroenteritis in humans. We recently illustrated a reduction-at-source approach to address the problem by orally administering C. jejuni-specific VH pentabodies to chickens, reducing their C. jejuni bacterial load by two to three orders of magnitude (Riazi et al., 2013). While our approach was successful, we believe that the isolation or engineering of more robust heavy-chain antibody variable domains (VHs) with greater gastrointestinal (GI) tract stability would eventually lead to a more efficacious strategy to control C. jejuni infections.

Stability is a key determinant of therapeutic antibody efficacy and strategies to increase antibody stability without compromising specificity and target binding affinity are desirable. For oral immunotherapeutics to succeed in the GI tract, they must resist proteolytic digestion. Monoclonal antibodies and larger antibody fragments (i.e. Fabs, scFvs) have shown limited success in the GI tract (Reilly et al., 1997; Singh et al., 2008; Hussack et al., 2011). Single-domain antibodies, particularly VHs from Camelidae, have the potential for oral immunotherapeutic development due to their high intrinsic stability and their amenability to screening, selection and engineering. Even so, orally delivered VHs still require considerable stabilization as demonstrated by the significant level of VH degradation in the stomach of pigs (Harmsen et al., 2006). A number of options exist to increase thermal, chemical and proteolytic stability of antibody fragments. These approaches include error-prone polymerase chain reaction (PCR) (Leung et al., 1989; Daugherty et al., 2000), DNA shuffling/random mutagenesis (Harmsen et al., 2006), site-directed mutagenesis (Wörn and Plückthun, 1998), phage library screening under selection pressures such as heat, acidic pH and proteases (Jespers et al., 2004; Famm et al., 2008; Dudgeon et al., 2012). Specific mutations introducing disulfide bonds have also been used to increase antibody stability (Young et al., 1995; Hagiwara et al., 2007; Saerens et al., 2008; Hussack et al., 2011, 2012; Kim et al., 2012, 2013).

Here, our aim was to improve the biophysical properties of a C. jejuni flagella-binding VH (Riazi et al., 2013) through two different approaches: by panning an error-prone PCR library in the presence of the major GI tract proteases and by disulfide-bond engineering. On their own, both mutations produced variants that were more stable compared with the parent VH. When the beneficial mutations were combined, a hyperstabilized VH with superior biophysical properties was produced; it retained function in terms of binding C. jejuni flagella and potently reduced C. jejuni motility in vitro.

Materials and methods

Construction of the mutant VH library by error-prone PCR

The vector pMED1 was first modified in order to remove tag sequences which were prone to protease digestion (data not shown). The new vector is named pMED6. For error-prone PCR, 10 ng of DNA coding for VH ‘FlagV1M’ (hereafter referred to as ‘V1’), a VH specific to C. jejuni flagella (Riazi et al., 2013), was used as the initial template and amplified in a 50-μl reaction using a random mutagenesis PCR kit (GeneMorph II Random Mutagenesis kit, Stratagene, Mississauga, ON, Canada) and
Protease panning of the V1 error-prone PCR library

Panning experiments were essentially performed as described previously (Arbabi-Ghahroudi et al., 1997, 2009a), except that rescued and amplified phage from the initial library and each round of panning were pre-treated with chicken GI tract fluid (Loc Carrillo et al., 2005) as well as pepsin, chymotrypsin and trypsin proteases. Three phage aliquots (125 μl each; 1 × 10^{12} phage particles) were prepared in 1 mM Tris–HCl buffer, pH 7.8. To the first phage aliquot, 12.5 μl GI tract chicken protease extract (diluted 1 in 10 in phosphate buffered saline, PBS) was added and incubated for 2 h at 37°C. The second phage aliquot was incubated with an equimolar mixture of chymotrypsin/trypsin (Roche, Laval, QC, Canada) (2.5 μM of each protease in Round 1, 7.5 μM in Round 2 and 10 μM in Rounds 3 and 4; final concentration) in 1 mM HCl plus 20 mM CaCl_{2} and incubated for 15 min in Round 1, 45 min in Round 2 and 60 min in Rounds 3 and 4. To the third phage aliquot, different concentrations of pepsin (Roche) were prepared in PBS and added along with 1/10 volume of 100 mM HCl, pH 2.0, to the 125 μl phage aliquot (2.5 μM in Round 1, 7.5 μM in Round 2 and 10 μM in Rounds 3 and 4; final concentrations). The protease reactions were stopped either by adding 12.5 μl of protease inhibitor cocktail (Roche) for chicken protease and trypsin/chymotrypsin or by adding 0.5 volume of 1 M Tris–HCl, pH 7.5, for pepsin. The protease-treated phage aliquots were mixed and used for panning. A total of four rounds of panning were performed against C. jejuni flagella as described previously (Arbabi-Ghahroudi et al., 2009a). Briefly, wells of a 96-well Maxisorp™ plate (Nunc, Rochester, NY) were coated with 15 μg of flagella or PBS (as a blank) overnight at 4°C. The wells were rinsed with PBS and blocked with PBS/1% (w/v) casein for 2 h at 37°C. Mixed protease-treated phage particles (100 μl) were added to the blocked wells and incubated for 2 h at 37°C. The wells were washed six times with PBS (2% (w/v) Tween-20) and six times with PBS. The bound phage were eluted with 0.1 M triethylamine, neutralized with 1 M Tris–HCl, pH 7.4, and incubated with exponentially growing E. coli TG1 cells. After 30 min of incubation at 37°C, the cells were superinfected with M13KO7 helper phage for an additional 15 min and grown in 2xYT/ampicillin/kanamycin overnight at 37°C. Amplified phage were purified and panning was continued for three more rounds following the same conditions, except that phage-protease digestion conditions were changed as described above, the antigen concentration was reduced to 12.5, 10 and 10 μg/well and washing was increased to 7, 10 and 12× with PBS and then 10 and 12× with PBS for the second, third and fourth rounds of panning, respectively. After four rounds of panning, 24 randomly picked colonies were subjected to colony PCR and the PCR fragments were sequenced. A total of nine flagella-specific V1Hs (F1, F3, F7, F10, F13, F14, F20, F22 and F23) with one to four nucleotide point mutations were identified. The mutant clones along with the parental V1 clone were grown and subjected to phage enzyme-linked immunosorbent assay (ELISA) screening as described previously (Arbabi-Ghahroudi et al., 2009a), except that 5 μg/ml of flagella was coated onto microtiter plates. Only the F7, F13, F20 and F23 clones showed positive signals and were selected for further characterization.

Expression and purification of soluble V1Hs

Genes encoding mutant V1Hs obtained from panning the error-prone PCR library against C. jejuni flagella were PCR-amplified from the pMED6 phagemid vector with BbsI1-V1H and BamHI-V1H primers (Table I). These PCR fragments were digested with BbsI and BamHI restriction endonucleases and ligated into the similarly digested pSFJ2H expression vector (Arbabi-Ghahroudi et al., 2009a). Upon ligation, all plasmids were transformed into electrocompetent E. coli TG1 and selected on LB agar plates containing ampicillin. Colonies were screened by colony PCR for inserts and the DNA sequenced. Small-scale expression was performed on individual clones and

Table I. Oligonucleotides used in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5′ → 3′)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>MJ7BACK</td>
<td>CATGTCATGGCGCTGGAGACTCGGCGCCAGCGCGCCCGCATGGC</td>
<td>Error-prone PCR</td>
</tr>
<tr>
<td>MJFOR 11</td>
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<td>Error-prone PCR</td>
</tr>
<tr>
<td>BbsI1-V1H</td>
<td>TATGAAAGACACCAGGCAGGGGAGTGATGAGG</td>
<td>Subcloning</td>
</tr>
<tr>
<td>BamHI-V1H</td>
<td>TGCTGGTAGTCTGGGAGGCGCTGGG</td>
<td>Subcloning</td>
</tr>
<tr>
<td>V1-DSB-for</td>
<td>TAGCAGATTAAGCTTCATAGCTGCAAGAAATAGCGGACACTACG</td>
<td>DSB cloning</td>
</tr>
<tr>
<td>V1-DSB-rev</td>
<td>GATGAATAGCTTCATAGCTGCAAGAAATAGCGGACACTACG</td>
<td>DSB cloning</td>
</tr>
<tr>
<td>F23-DSB-for</td>
<td>TATGAAAGACACCAGGCAGGGGAGTGATGAGG</td>
<td>DSB cloning</td>
</tr>
<tr>
<td>F23-DSB-rev</td>
<td>GATGAATAGCTTCATAGCTGCAAGAAATAGCGGACACTACG</td>
<td>DSB cloning</td>
</tr>
</tbody>
</table>
only clone F23 was found to have an expression level comparable with that of the parental V1 clone.

VHs V1 and F23 were expressed using a 5 day minimal media method (Baral and Arbabi-Ghahroudi, 2012). After induction of protein expression, cell cultures were harvested at 6000 x g (30 min, 4°C), the supernatant discarded and the periplasmic contents extracted from the cell pellet. Briefly, cell pellets containing VHs were resuspended in 20 ml of ice-cold TES (0.2 M Tris–HCl, pH 8.0), 20% (w/v) sucrose, 0.5 mM EDTA and incubated on ice for 30 min. Next, 30 ml of ice-cold 1/8 TES was added, the mixture was incubated for an additional 30 min on ice, and the slurry centrifuged at 8000 x g (30 min, 4°C). The resulting supernatant containing VH was dialyzed overnight into buffer A (10 mM HEPES, pH 7.0, 500 mM NaCl) and VHs were purified by immobilized metal-ion affinity chromatography (IMAC) as described (Arbabi-Ghahroudi et al., 2009a). Eluted fractions were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting (data not shown) before being dialyzed into PBS. VH concentrations were determined by absorbance measurements at 280 nm using theoretical molecular mass and extinction coefficients calculated with the ExPASy ProtParam Tool (http://expasy.org/tools/protparam.html) (Page et al., 1995).

Construction of disulfide-bond VH variants

Cysteine mutations were introduced at positions 54 and 78 of V1 and F23 VHs, for the generation of a non-canonical disulfide bond, using splice-overlap extension PCR essentially as described (Hussack et al., 2011). To create the V1-DSB VH, which contains two disulfide bonds, the overlap PCR extension fragments were amplified using Bbs1-V1H/V1-DSB-rev and V1-DSB-for/BamH1-V1H primer sets (Table I). Likewise, the F23-DSB VH was amplified by melting the DNA encoding F23 with F23-DSB-for/V1-DSB-rev and V1-DSB-for/BamH1-V1H primer sets (Table I). All DNA constructs were cloned into the pSJF2H expression vector, transformed into E. coli TG1 and expressed/purified as described for V1 and F23.

**Size-exclusion chromatography and surface plasmon resonance analyses**

Size-exclusion chromatography (SEC) was performed prior to surface plasmon resonance (SPR) analysis using a Superdex™ 75 column and 50 μM of VH, as described previously (Hussack et al., 2011). Chromatograms were normalized as described previously (Kim et al., 2012). Prior to SPR analysis on a Biacore 3000 (GE Healthcare, Baie-d’Urfe, QC, Canada), flagella from C. jejuni strain 81–176 was isolated (Riazi et al., 2013), and biotinylated using an EZ-Link Sulfo-NHS-Biotinylation kit according to the manufacturer’s instructions (Fisher Scientific, Ottawa, ON, Canada). Uncoupled biotin was removed by dialysis against 1000 x volume of PBS for a total of eight times. For binding analysis, 2000 resonance units (RU) of biotinylated flagella were immobilized onto a CAP sensor chip (GE Healthcare) previously loaded with the biotin CAPture reagent (50 μg/ml in HBS-EP buffer; GE Healthcare). Monomeric VHs eluted from the SEC column were injected over the immobilized flagella at concentrations ranging from 25–400 nM, using multiple injections of increasing concentrations and single-cycle kinetic analysis. The running buffer for all SPR experiments was HBS-EP (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, pH 7.4, 0.005% surfactant P20; GE Healthcare). The surface was stripped after each VH using regeneration buffers (8 M guanidine hydrochloride and 1 M NaOH) and a fresh biotinylated flagella surface was prepared as described above.

**Thermal unfolding analysis of VHs**

VHs V1H were determined by circular dichroism measurements at pH values 7.3 and 2.0 using a Jasco J-815 spectrometer (Jasco, Easton, MD) by following VH unfolding at 215 nm, essentially as described (Hussack et al., 2011). The only exception to the previously described protocol (Hussack et al., 2011) was that circular dichroism measurements were taken every 0.2° C from 25 to 96° C. In the case of V1 VH, 10 μg/ml was used for Tm determination at pH 7.3 because of aggregation at high temperatures using 50 μg/ml.

**In vitro protease digestions of VHs**

VHs were subjected to protease digestion assays with the major GI proteases pepsin (Sigma, Oakville, ON, Canada), sequencing-grade trypsin (Roche) and sequencing-grade chymotrypsin (Roche). VH digestions were performed and quantified exactly as described (Hussack et al., 2011) and analyzed by SDS–PAGE thereafter. A total of three independent protease digestions were performed on each VH and each of these independent digestions was performed in duplicates.

In addition to individual protease digestions, a sequential digestion reaction was performed in which VHs (50 μg) were first digested with pepsin (10 μg/ml, 37°C, pH 2.0) for 15 or 30 min, followed by digestion with trypsin + chymotrypsin (10 μg/ml for each, 37°C, pH 7.4) for 15 or 30 min, in a total volume of 50 μl. After the pepsin digestion, the pH of the reaction was neutralized with 1 M NaOH to stop the reaction. After the trypsin + chymotrypsin digestion, protease inhibitor cocktail (Sigma) was added to stop the reaction. Sequentially digested VHs were compared with non-treated controls by SDS–PAGE and analyzed for functionality in a C. jejuni motility assay (see below).

**Campylobacter jejuni motility assay**

Campylobacter jejuni motility assays were performed as described previously (Kalmokoff et al., 2006). VHs, at a final concentration of 1 μg/μl, were incubated with C. jejuni (5 x 10^4 colony-forming units) at room temperature for 30 min. The mixtures were plated, as described in Waseh et al. (2010), in the center of a Petri dish containing Müller–Hinton agar (0.4%) and incubated at 37°C under microaerobic conditions (5% O2, 10% CO2 and 85% N2). Bacterial motility was determined by measuring the diameter of the circle produced by the growing bacteria at 24 h after plating the bacteria.

**Results**

**Construction of the mutant V1 library by error-prone PCR and protease panning**

The VH FlagV1M (referred to as ‘V1’ in this work) was isolated from a plaque-displayed immune llama library against C. jejuni flagella (Riazi et al., 2013). Before construction of a VH error-prone PCR library to select for protease-resistant variants, we removed the His/HA tags from the pMED1 phased vector (Arbabi-Ghahroudi et al., 2009b), generating pMED6 (Fig. 1A). The His/HA tags were previously shown
mutations were introduced into the V1 VHH gene by error-prone PCR method (Leung et al., 1989; Stemmer, 1994) to introduce random mutations with the purpose of isolating protease-resistant mutants. A similar method has been successfully used for affinity maturation of conventional IgG antibodies as well as scFvs and V NARs (Miyazaki et al., 1992; Fennell et al., 2000; Nuttall et al., 2004; Fennell et al., 2010). For library construction, random mutations were introduced into the V1 VHH gene by error-prone PCR and the amplified PCR products were used to construct the mutant library with an approximate size of 2 x 10^9 transformants. Sequencing data from randomly selected colonies demonstrated the presence of point mutations within the V1 VHH amino acid sequence at the rate of three to five nucleotide substitutions per V1 VHH gene (data not shown). For panning, and in order to select V1 VHHs resisting the chicken GI tract environment, the rescued phage antibodies were pre-incubated with either chicken GI tract fluids harboring various proteases or the major GI proteases pepsin, trypsin and chymotrypsin (Fig. 1B). The filamentous phages (f1, fd and M13) are known to be sensitive to proteolytic cleavage (data not shown). The new pMED6 phagemid vector contained an amber stop codon 4 nucleotides downstream of the 5' SfiI restriction endonuclease site where the previous His6/HA tag was located. We adopted an error-prone PCR method (Leung et al., 1989; Stemmer, 1994) to introduce random mutations with the purpose of isolating protease-resistant mutants. A similar method has been successfully used for affinity maturation of conventional IgG antibodies as well as scFvs and V NARs (Miyazaki et al., 1992; Fennell et al., 2000; Nuttall et al., 2004; Fennell et al., 2010). For library construction, random mutations were introduced into the V1 VHH gene by error-prone PCR and the amplified PCR products were used to construct the mutant library with an approximate size of 2 x 10^9 transformants. Sequencing data from randomly selected colonies demonstrated the presence of point mutations within the V1 VHH amino acid sequence at the rate of three to five nucleotide substitutions per V1 VHH gene (data not shown). For panning, and in order to select V1 VHHs resisting the chicken GI tract environment, the rescued phage antibodies were pre-incubated with either chicken GI tract fluids harboring various proteases or the major GI proteases pepsin, trypsin and chymotrypsin (Fig. 1B). The filamentous phages (f1, fd and M13) are known to be resistant to most GI tract restriction endonuclease sites, and the amber stop codon (underlined). The multi-cloning site of the new phagemid vector, pMED6, is shown. (B) Work-flow diagram highlighting the construction of the V1 VHH error-prone PCR library, protease treatment of phages and panning scheme. (C) Phage ELISA on nine V1 VHHs isolated from panning the V1 error-prone PCR library with protease treatment. Campylobacter jejuni strain 81–176 flagella was coated in microwells and binding of phage-displayed V1 VHHs was detected with anti-M13-IgG conjugated to HRP. Asterisks denote the four clones which showed comparable binding signals to the parent V1 VHH, V1. (D) Sequences of the nine V1 VHHs tested by phage ELISA in (C). Grayed areas highlight the hypervariable loops (IMGT numbering; http://www.imgt.org/).

**Construction of disulfide-engineered variant V1 VHHs and SEC analysis**

In addition to the parental V1 VHH and the F23 V1 VHH, which was isolated form protease panning of the error-prone PCR library, an engineered variant of each V1 VHH was produced with a second disulfide bond by mutating Ala54 and Ile78 to Cys54 and Cys78, giving V1-DSB and F23-DSB (Fig. 2A and B). All V1 VHHs were expressed and purified by IMAC and shown to be pure by SDS-PAGE (Fig. 2C). All ran at their expected molecular masses and there were no signs of higher-order multimers on the non-reducing SDS-PAGE gel. V1 VHH expression yields ranged from 9.5 mg (V1-DSB) to 21 mg (F23) per liter of bacterial culture (Table II), which is consistent with our previous findings that the Cys54-Cys78 disulfide bond compromises V1 VHH expression (Hussack et al., 2011). SEC chromatograms suggested that all of the V1 VHHs were monomeric (Fig. 2E). Intact mass and fingerprinting analysis was not performed to confirm the formation of the Cys54-Cys78 disulfide bond within the engineered variants (Hussack et al., 2011); however, the significant increase in V1 VHH T<sub>m8</sub> (see below) and reduction in yields strongly suggest the introduced disulfide bond was successfully formed, as shown before for several other V1 VHHs and V1 domains harboring the identical Cys54-Cys78 mutations (Hussack et al., 2011; Kim et al., 2012, 2013).

**Binding and thermal unfolding analyses of V1 VHHs**

The affinity of all 4 V1 VHHs was determined by SPR using single-cycle kinetics. The sensorgrams and affinities of V1, V1-DSB, F23 and F23-DSB are shown (Fig. 2D) and reported in Table II. All of the V1 VHH binding data fit a 1:1 binding model and V1 VHHs retained high affinity binding to *C. jejuni*

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Fig. 1. Isolation of protease-resistant V1 VHH V1 variants. (A) Before constructing the error-prone PCR library based on *C. jejuni* flagella-specific V1 VHH V1, the phagemid vector pMED1 was modified to remove the His6/HA tags that were previously shown to be susceptible to proteolytic cleavage. DNA encoding the His6/HA tag was removed from the sequence between the 5' SfiI site (bold and italics) and the amber stop codon (underlined). The multi-cloning site of the new phagemid vector, pMED6, is shown. (B) Work-flow diagram highlighting the construction of the V1 VHH error-prone PCR library, protease treatment of phages and panning scheme. (C) Phage ELISA on nine V1 VHHs isolated from panning the V1 error-prone PCR library with protease treatment. *Campylobacter jejuni* strain 81–176 flagella was coated in microwells and binding of phage-displayed V1 VHHs was detected with anti-M13-IgG conjugated to HRP. Asterisks denote the four clones which showed comparable binding signals to the parent V1 VHH, V1. (D) Sequences of the nine V1 VHHs tested by phage ELISA in (C). Grayed areas highlight the hypervariable loops (IMGT numbering; http://www.imgt.org/).
flagella, with similar $K_D$s, ranging from 23.9 nM (V1) to 17.3 nM (F23-DSB), demonstrating no significant changes in binding affinity upon introducing mutations in FR1 or addition of an extra disulfide bridge in the V1H domain.

The $T_m$ of all four V1Hs was determined by circular dichroism spectroscopy at pH values 7.3 and 2.0. Unfolding curves with a single-phase transition were obtained for all V1Hs at pH 2.0. Before performing unfolding experiments, V1Hs were incubated for 2 h at pH 2.0. At this pH, V1 was completely unfolded at the starting temperature (25°C) and a $T_m$ could not be determined (Fig. 3). V1-DSB on the other hand was folded at 25°C at pH 2.0 and had a $T_m$ of 30.5 ± 0.3°C, illustrating the significant impact the second disulfide bond has on V1H stability. F23 was partially unfolded at 25°C at pH 2.0 and a $T_m$ of 44.6 ± 0.1°C was determined, an improvement from the parental V1 V1H which was completely unfolded under these conditions. This result underscores the success of the protease-panning strategy in selecting for more stable binders and with a higher $T_m$. Similar to pH 7.3, the clone with the highest $T_m$ at pH 2.0 was F23-DSB, with a $T_m$ of 44.6 ± 0.1°C, suggesting that the effects of the stabilizing mutations in F23 and the effects of the second disulfide bond were partially additive to create the hyperstabilized F23-DSB V1H.

### In vitro protease digestion of V1Hs

The resistance of V1, V1-DSB, F23 and F23-DSB to the major GI proteases was determined (Fig. 4; Table II). Both V1 and F23 V1Hs were susceptible to pepsin degradation, with 22.3 ± 8.1 and 6.8 ± 3.6% of V1Hs remaining intact after 60 min exposure to pepsin, respectively. The disulfide-engineered variants were considerably more resistant to pepsin, with V1-DSB

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**Table II. Biophysical properties of C. jejuni flagella-specific V1Hs.**

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<thead>
<tr>
<th>V1H</th>
<th>Yield (mg/l)</th>
<th>$k'_e$ (M$^{-1}$ s$^{-1}$)</th>
<th>$k_e$ (s$^{-1}$)</th>
<th>$K_m$ (nM)</th>
<th>$T_m$, pH 7.3 (°C)</th>
<th>$T_m$, pH 2.0 (°C)</th>
<th>Peptin res. (%)</th>
<th>Trypsin res. (%)</th>
<th>Chymotrypsin res. (%)</th>
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<tr>
<td>V1</td>
<td>17.0</td>
<td>2.36 x 10$^5$</td>
<td>5.63 x 10$^{-3}$</td>
<td>29.3</td>
<td>61.7 ± 0.4</td>
<td>Nd</td>
<td>22.3 ± 8.1</td>
<td>84.4 ± 1.8</td>
<td>52.9 ± 6.5</td>
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<tr>
<td>V1-DSB</td>
<td>9.5</td>
<td>1.53 x 10$^5$</td>
<td>2.80 x 10$^{-3}$</td>
<td>18.2</td>
<td>79.1 ± 0.1</td>
<td>42.4 ± 0.1</td>
<td>43.4 ± 3.8</td>
<td>49.5 ± 6.7</td>
<td>95.9 ± 4.1</td>
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<tr>
<td>F23</td>
<td>21</td>
<td>2.29 x 10$^5$</td>
<td>4.26 x 10$^{-3}$</td>
<td>18.6</td>
<td>72.3 ± 0.8</td>
<td>30.5 ± 0.3</td>
<td>100.5 ± 6.7</td>
<td>101.1 ± 4.7</td>
<td>85.4 ± 3.3</td>
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<tr>
<td>F23-DSB</td>
<td>16.5</td>
<td>1.65 x 10$^5$</td>
<td>2.86 x 10$^{-3}$</td>
<td>17.3</td>
<td>80.2 ± 0.4</td>
<td>44.6 ± 0.1</td>
<td>96.9 ± 15.8</td>
<td>49.1 ± 15.4</td>
<td>90.9 ± 4.1</td>
</tr>
</tbody>
</table>

$^a$10 μg/ml of V1H used for circular dichroism due to the aggregation of V1 at 50 μg/ml.
$^b$Digestions performed using 100 μg/ml of protease.
$^c$Digestions performed using 10 μg/ml of protease.
Nd: not determined since the protein denatured at pH 2.0, 25°C, res., resistance.
showing complete resistance (100.5 ± 6.7%) at a pepsin concentration of 100 μg/ml. F23-DSB was also very resistant to pepsin, with 96.9 ± 15.8% of the VHH remaining intact after 60 min. When digested with trypsin for 60 min, F23 was completely resistant (101.1 ± 4.7%) followed by V1 (84.4 ± 1.8%), F23-DSB (49.1 ± 15.4%) and V1-DSB (41.3 ± 2.7%). It is obvious that the addition of the second disulfide bond between Cys54–Cys78 reduces trypsin resistance for both clones here, a result that is similar to previous findings for some C. difficile toxin A specific VHHs (Hussack et al., 2011). The significant reduction in trypsin resistance of F23-DSB suggests that structural changes in the VHH domain may lead to the exposure of trypsin-sensitive sites (i.e. Lys and Arg) upon the addition of the second disulfide bridge. When digested with chymotrypsin for 60 min, F23-DSB displayed the highest resistance (90.9 ± 4.1%) followed by F23 (85.4 ± 3.3%), V1 (52.9 ± 6.5%) and V1-DSB (49.5 ± 6.7%). Therefore, in the case of these VHHs, the second disulfide bond does not affect chymotrypsin resistance but does decrease trypsin resistance. Collectively, these data suggest that the F23 variant isolated from protease-panning was selected for very high trypsin and chymotrypsin resistance, and that the strongest combined resistance for all three proteases is the F23-DSB variant.

Sequential protease treatment of V1Hs

To determine whether the two strategies used to isolate robust, hyperstabilized V1Hs were effective in a biological setting, we exposed all four V1Hs to a sequential protease digestion scheme reminiscent of the GI tract (Fig. 5A, upper panel). Specifically, we treated V1Hs with pepsin at pH 2.0 followed by trypsin and chymotrypsin at pH 7.3 for 15 or 30 min each. Analysis of the sequential digestions and non-treated controls by SDS–PAGE is shown (Fig. 5A, lower panel). Near complete digestion of V1 is evident at 15 and 30 min relative to control V1 (compare ‘V1’ with ‘V1(15)’ and ‘V1(30)’). F23 was more resistant than V1, with a strong band representing intact V HH present after the sequential 15 min digest (compare ‘F23’ with ‘F23(15)’). After 30 min, a near complete digestion of F23 was evident. Conversely, both disulfide-bond variants were strongly resistant to the sequential protease digestions, even after 30 min of treatment (compare ‘V1-DSB’ with ‘V1-DSB(30)’ and compare ‘F23-DSB’ with ‘F23(30)’). Densitometry analysis of the bands revealed 74.5% of F23-DSB VHH intact after the sequential 30 min digest compared with 47.6% of V1-DSB VHH intact after the same treatment.

Campylobacter jejuni motility assays

Finally, we tested the C. jejuni motility inhibiting capacity of the V1H variants. All V1Hs were effective in inhibiting the growth and spread of C. jejuni after incubation for 24 h, with essentially the same efficacy (Fig. 5B and C). We then used the sequential protease-treated V1Hs (Fig. 5A) in the same motility assay to confirm the disulfide-bond variant V1Hs retained their functionality. The protease-treated V1 and F23 V1Hs were almost entirely degraded after 15 or 30 min incubation conditions, and as expected, did not inhibit motility.
On the other hand, the growth inhibiting activity of V1-DSB and F23-DSB mutants was basically unaffected by the protease treatment, further confirming that the protease-treated V1Hs remain largely intact (Fig. 5A) and active in settings reminiscent of the GI tract.

Discussion

In this study, we report the generation of a hyperstabilized V1H against C. jejuni flagella with high thermal and proteolytic stability. This was achieved by combining an error-prone PCR approach and panning under protease pressure with the introduction of cysteine residues at positions 54 and 78 in the FR1 region of the V1H scaffold, as evidenced by the lowered trypsin resistance of F23-DSB, leading to the speculation that one or more potential trypsin cleavage sites in FRs (namely, R18, R19, K43, R45, K64 and K86) are more accessible for proteolytic digestion. This is consistent with our previous work on other V1Hs (Hussack et al., 2011; Kim et al., 2012, 2013), and led to the generation of the hyperstable V1H F23-DSB. However, the addition of a second disulfide bond leads to minor structural changes when introduced into the F23 V1H scaffold, as evidenced by the lowered trypsin resistance of F23-DSB, leading to the speculation that one or more potential trypsin cleavage sites in FRs (namely, R18, R19, K43, R45, K64 and K86) are more accessible for proteolytic digestion. This is consistent with our previous work on other V1Hs (Hussack et al., 2011). While it is obvious that the second disulfide bond leads to structural changes, these changes must be minor, and possibly restricted to influencing the framework regions only, because the antigen binding affinity of both V1Hs containing the second disulfide bond are nearly identical to that of the parental scaffolds. In the future, strategies to increase the trypsin resistant properties of F23-DSB could include the replacement of the framework residues noted above and other trypsin-sensitive residues (Frenken et al., 1993) by site-specific mutations or re-applying the error-prone PCR approach followed by panning under protease pressure against the same target antigen (as shown in this study).

The antibody engineering approach used here produced robust binding agents with favorable biophysical properties. Currently, the protein-engineered F23-DSB V1H is being
studied for the prevention or significant reduction of *C. jejuni* colonization in the chicken GI tract.

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### References


