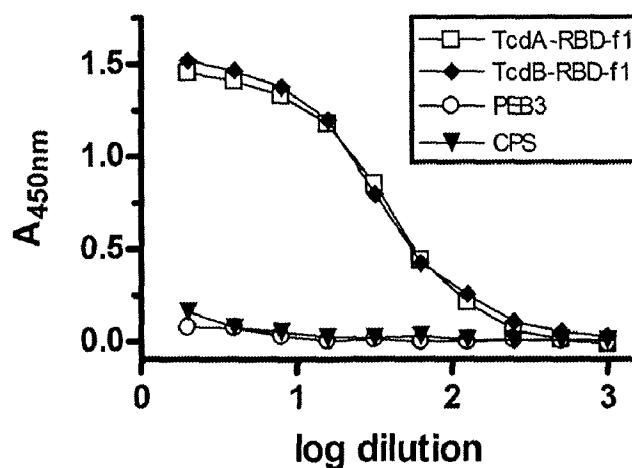




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(54) **Titre : ANTICORPS SPECIFIQUES DE CLOSTRIDIUM DIFFICILE ET LEURS APPLICATIONS**
(54) **Title: CLOSTRIDIUM DIFFICILE-SPECIFIC ANTIBODIES AND USES THEREOF**



(57) **Abrégé/Abstract:**

The present invention is directed to Clostridium difficile toxin-specific antibodies, compositions, and uses thereof. The anti-toxin antibodies may be specific for either TcdA or TcdB. The invention also includes methods of treating a Clostridium difficile infection, methods of capturing Clostridium difficile toxins, and methods of detecting Clostridium difficile toxins.

ABSTRACT

The present invention is directed to *Clostridium difficile* toxin-specific antibodies, compositions, and uses thereof. The anti-toxin antibodies may be specific for either TcdA or TcdB. The invention also includes methods of treating a *Clostridium difficile* infection, methods of capturing *Clostridium difficile* toxins, and methods of detecting *Clostridium difficile* toxins.

CLOSTRIDIUM DIFFICILE-SPECIFIC ANTIBODIES AND USES THEREOFFIELD OF THE INVENTION

The present invention relates to *Clostridium difficile*-specific antibodies and uses thereof. More specifically, the present invention relates to *Clostridium difficile* toxin-specific antibodies and
5 uses thereof.

BACKGROUND OF THE INVENTION

Clostridium difficile is a Gram-positive, anaerobic, endospore-forming gastrointestinal pathogen responsible for *C. difficile*-associated disease (CDAD) in humans and animals with symptoms ranging in severity from mild cases of antibiotic-associated diarrhea to fatal
10 pseudomembranous colitis (Rupnik et al, 2009; Leffler and Lamont, 2009; Songer, 2004; Kelly et al, 1994). Each year in North America, 1-3 % of hospitalized patients receiving antibiotics become infected with *C. difficile*, leading to thousands of deaths and over \$1 billion in associated costs to the health-care system (Wilkins and Lyster, 2003; Kyne et al, 2002; Kelly et al, 1994). *C. difficile* produces two primary virulence factors, toxin A (TcdA) and toxin B (TcdB),
15 which are large (308 kDa and 269 kDa, respectively), single-subunit exotoxins composed of a catalytic, a translocation and a cell-receptor binding domain (RBD) (Jank and Aktories, 2008; Jank et al, 2007). Recently it was suggested TcdB is solely responsible for *C. difficile* virulence (Lyras et al, 2009), although earlier studies have shown both anti-TcdA and anti-TcdB monoclonal antibodies (mAbs) were required for full protection of hamsters from CDAD
20 (Babcock et al, 2006; Kink and Williams, 1998) and anti-TcdA mAbs were required for protection in mice (Corthier et al, 1991).

The current approach for treating most CDAD infections involves administration of antibiotics, most commonly metronidazole or vancomycin (Leffler and Lamont, 2009). Antibiotic treatment places selection pressure on the organism, can lead to antibiotic resistance, and suppresses
25 or eliminates beneficial commensal microbes. However, there are several other emerging challenges warranting the development of novel therapeutics. First, there is no acute CDAD treatment targeting TcdA/B. These toxins are responsible for loss of epithelial barrier function in the colon by disrupting tight junctions and increasing membrane permeability, causing diarrhea and promoting severe inflammation (Rupnik et al, 2009; Jank and Aktories, 2008).
30 Second, hypervirulent strains of *C. difficile*, such as the NAP1/027 isolate, over-express TcdA and TcdB (Warny et al, 2005) and have been associated with increased mortality rates and disease severity (O'Connor et al, 2009; Pépin et al, 2005). Third, an estimated 20-25% of

patients suffering from CDAD experience symptomatic relapse after the initial infection is cleared, with 45% of these patients prone to subsequent relapses (Johnson, 2009). Taken together, there is a need for non-antibiotic based reagents which target and inhibit TcdA and TcdB for CDAD therapy.

- 5 Individuals who are asymptomatic *C. difficile* carriers and patients who experience mild cases of CDAD tend to possess high anti-toxin A titers (Kyne et al, 2001; Kyne et al, 2000; Warny et al, 1994; Viscidi et al, 1983). Conversely, patients susceptible to relapsing *C. difficile* infection have low anti-TcdA immunoglobulin titers, specifically IgM, IgG2 and IgG3 isotypes (Katchar et al, 2007; Kyne et al, 2001). TcdA-neutralizing secretory IgA antibodies are also thought to play
10 a role in regulating CDAD severity (Johal et al 2004; Kelly et al 1992). Therefore, the introduction of anti-toxin antibodies to patients suffering from severe *C. difficile* infection may be a therapeutically useful approach.

A limited number of animal and human studies have illustrated the effectiveness of anti-toxin Abs for treatment of CDAD. Babcock et al (2006) intravenously administered anti-TcdA and
15 anti-TcdB mAbs to hamsters and found a significant reduction in hamster mortality in prophylactic, primary disease and relapse models when both anti-toxin mAbs were administered. A recently completed clinical trial involving these two humanized mAbs appears promising (Lowy et al, 2010). In another study, intravenous administration of anti-TcdA mAbs raised against the RBD followed by oral challenge with *C. difficile* resulted in protection of mice
20 (Corthier et al, 1991). Elsewhere, a toxoid vaccine given by the intraperitoneal route to hamsters conferred protection against oral *C. difficile* challenge (Giannasca et al, 1999) and mice vaccinated with DNA encoding the TcdA RBD resulted in full protection from oral TcdA challenge (Gardiner et al, 2009). In humans, a number of uncontrolled studies have reported intravenous immunoglobulin (IVIG) therapy to be successful for the treatment of severe CDAD
25 (Juang et al, 2007; Hassoun and Ibrahim, 2007; McPherson et al, 2006; Wilcox, 2004; Salcedo et al, 1997; Leung et al, 1991). IVIG involves administration of high concentrations (150 – 400 mg/kg) of human immunoglobulins from healthy donors which are thought to contain neutralizing anti-toxin antibodies as an estimated 60% of healthy adults have detectable TcdA- and TcdB-specific serum IgG antibodies (Viscidi et al, 1983).

- 30 Given that *C. difficile* toxins rely on attachment to epithelial cells for entry (Jank and Aktories, 2008; Jank et al, 2007), neutralizing the toxins within the lower gastrointestinal tract with antibodies may block the first step in CDAD pathogenesis. In animals, orally administered bovine immunoglobulin concentrate (BIC) containing TcdA and TcdB neutralizing IgGs were able to prevent hamster mortality when used as a prophylactic (Lyerly et al, 1991) and
35 protected rats from the enterotoxic effects of TcdA *in vivo* (Kelly et al, 1996). Chicken IgY

antibodies specific for toxin RBDs were shown to reduce hamster mortality when administered orally to infected animals (Kink and Williams, 1998). In humans, there have been limited reports on CDAD therapy with orally delivered Abs. Tjellström et al (1993) reported the successful treatment of a 3 ½ year old boy suffering from severe CDAD with IgA antibody orally. Warny et al (1999) and Kelly et al (1997) examined the passage of anti-toxin bovine IgG through the human gastrointestinal tract and found a significant reduction in IgG activity, likely due to proteolytic degradation within the upper gastrointestinal tract. The limited success of both oral and systemic anti-toxin immunotherapy in clinical settings has likely been hampered by the high immunoglobulin dose requirements (150 – 400 mg/kg), the associated costs of these doses, and a lack of published clinical data showing the effectiveness of these treatments.

Despite such advances, there remains a need in the art for a safe and effective therapeutic for treating *C. difficile*-associated disease as well as for sensitive and effective reagents for the detection of toxins A and B, the factors responsible for *C. difficile*-associated disease.

15 SUMMARY OF THE INVENTION

The present invention relates to *Clostridium difficile*-specific antibodies and uses thereof. More specifically, the present invention relates to *Clostridium difficile* toxin-specific antibodies and uses thereof.

The present invention provides an isolated or purified antibody or fragment thereof, comprising

20 a sequence of complementarity determining region (CDR) 1 selected from GRTFNTLS (SEQ ID NO:1); GRTFSMYR (SEQ ID NO:2); GRTLSSYI (SEQ ID NO:3); GRTFSMDP (SEQ ID NO:4); IRSFSNRN (SEQ ID NO:5); and ERTFSRYP (SEQ ID NO:6);

a sequence of CDR2 selected from VSRSGGST (SEQ ID NO:7); ITRNGSST (SEQ ID NO:8); ISRRGGNS (SEQ ID NO:9); GSSTGRIT (SEQ ID NO:10); ISWGGGST (SEQ ID NO:11); and ISSTGTST (SEQ ID NO:12); and

25 a sequence of CDR3 selected from AAAATKSNTTAYRLSFDY (SEQ ID NO:13); AATSGSSYLDAAHVYDY (SEQ ID NO:14); AADGSVAGWGRRSVSVSSYDY (SEQ ID NO:15); AAPYGANWYRDEYAY (SEQ ID NO:16); AAefGHNIATSSDEYDY (SEQ ID NO:17); and AVNSQRTRLQDPNEYDY (SEQ ID NO:18),

30 wherein the antibody or fragment thereof is specific for TcdA. The isolated or purified antibody or fragment thereof as described above may be selected from the group consisting of:

- an isolated or purified antibody or fragment thereof comprising a sequence of CDR 1 of GRTFNTLS (SEQ ID NO:1); CDR2 of VSRSGGST (SEQ ID NO:7); and CDR3 of AAAATKSNTTAYRLSFDY (SEQ ID NO:13);
- 5 an isolated or purified antibody or fragment thereof comprising a sequence of CDR 1 of GRTFSMYR (SEQ ID NO:2); CDR2 of ITRNGSST (SEQ ID NO:8); and CDR3 of AATSGSSYLDAAHVYDY (SEQ ID NO:14);
- an isolated or purified antibody or fragment thereof comprising a sequence of CDR 1 of GRTLSSYI (SEQ ID NO:3); CDR2 of ISRRGGNS (SEQ ID NO:9); and CDR3 of AADGSVAGWGRRSVSVSSYDY (SEQ ID NO:15);
- 10 an isolated or purified antibody or fragment thereof comprising a sequence of CDR 1 of GRTFSMDP (SEQ ID NO:4); CDR2 of GSSTGRTT (SEQ ID NO:10); and CDR3 of AAAPYGANWYRDEYAY (SEQ ID NO:16);
- an isolated or purified antibody or fragment thereof comprising a sequence of CDR 1 of IRSFSNRN (SEQ ID NO:5); CDR2 of ISWGGGST (SEQ ID NO:11); and CDR3 of
- 15 AAIEFGHNIATSSDEYDY (SEQ ID NO:17); or
- an isolated or purified antibody or fragment thereof comprising a sequence of CDR 1 of ERTFSRYP (SEQ ID NO:6); CDR2 of ISSTGTST (SEQ ID NO:12); and CDR3 of AVNSQRTRLQDPNEYDY (SEQ ID NO:18).

The isolated or purified antibody or fragment thereof described above may comprise a

20 sequence selected from the group consisting of:

- QVKLEESGGGLVQAGGSLRLSCAASGRTFNTLSMGWFRQAPGKEREFVAAVSRSGGST
YYADSVKGRFTISRDNKNTVYLQMNSLKPEDTAVYYCAAATKSNTTAYRLSFDYWGQG
TQVTVSS (SEQ ID NO:34);
- 25 QVKLEESGGGLVQAGGSLRLSCAASGRTFSMYRMGWFRQAPGKEREFVGVITRNGSSTY
YADSVKGRFTISRDNKNTVYLQMNSLKPEDTALYYCAATSGSSYLDAAHVYDYWGQGT
QVTVSS (SEQ ID NO:35);
- QVKLEESGGGLVQPGGSLRLSCAASGRTLSSYIVAWFRQAPGKEREFVAGISRRGGNSAY
VESVKGRFTISRDNKNTVYLQMNSLKPEDTAVYYCAADGSVAGWGRRSVSVSSYDYWG
QGTQVTVSS (SEQ ID NO:36);

- QVQLVESGGGLAQAGGSLRLSCAASGRTFSMDPMAWFRQPPGKEREFVAAGSSTGRTT
YYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAAAPYGANWYRDEYAYWGQGT
QVTVSS (SEQ ID NO:37);
- 5 QVQLVESGGGLVQAGGSLRLSCAASIRSFSNRNMGWFRQPPGKEREFVAGISWGGGST
RYADSVKGRFTISRDNAKKTVYLQMNSLKPEDTAVYYCAAEEFGHNIATSSDEYDYWGQGT
QVTVSS (SEQ ID NO:38);
- QVKLEESGGGLVQAGGSLRLSCAASERTFSRYPVAWFRQAPGAEREFVAVISSTGTSTYY
ADSVKGRFTISRDNAKVTVYLQMNNLKREDTAVYFCAVNSQRTRLQDPNEYDYWGQGTQ
VTVSS (SEQ ID NO:39);
- 10 QVKLEESGGGLVQAGGSLRLSCAASGRTFNTLSMGWFRQAPGKEREFVCAVSRSGGST
YYADSVKGRFTCSRDNAKNTVYLQMNSLKPEDTAVYYCAAATKSNTTAYRLSFDYWGG
GTQVTVSS (SEQ ID NO:45);
- QVKLEESGGGLVQAGGSLRLSCAASGRTFSMYRMGWFRQAPGKEREFVCVITRNGSSTY
YADSVKGRFTCSRDNAKNTVYLQMNSLKPEDTALYYCAATSGSSYLDAAHVYDYWGQGT
15 QVTVSS (SEQ ID NO:46);
- QVKLEESGGGLVQPGGSLRLSCAASGRTLSSYIVAWFRQAPGKEREFVCGISRRGGNSA
YVESVKGRFTCSRDNAKNTVYLQMNSLKPEDTAVYYCAADGSVAGWGRRSVSVSSYDY
WGQGTQVTVSS (SEQ ID NO:47);
- QVQLVESGGGLAQAGGSLRLSCAASGRTFSMDPMAWFRQPPGKEREFVCAGSSTGRTT
20 YYADSVKGRFTCSRDNAKNTVYLQMNSLKPEDTAVYYCAAAPYGANWYRDEYAYWGQG
TQVTVSS (SEQ ID NO:48);
- QVQLVESGGGLVQAGGSLRLSCAASIRSFSNRNMGWFRQPPGKEREFVCGISWGGGST
RYADSVKGRFTCSRDNAKKTVYLQMNSLKPEDTAVYYCAAEEFGHNIATSSDEYDYWGQG
TQVTVSS (SEQ ID NO:49); and
- 25 QVKLEESGGGLVQAGGSLRLSCAASERTFSRYPVAWFRQAPGAEREFVCVISSTGTSTYY
ADSVKGRFTCSRDNAKVTVYLQMNNLKREDTAVYFCAVNSQRTRLQDPNEYDYWGQGT
QVTVSS (SEQ ID NO:50),

or a sequence substantially identical thereto.

- In another aspect, the present invention provides an isolated or purified antibody or fragment
30 thereof, comprising

a sequence of complementarity determining region (CDR) 1 selected from GNIFSINT (SEQ ID NO:19); GRTASGYG (SEQ ID NO:20); GRTFSSGV (SEQ ID NO:21); GLSRYA (SEQ ID NO:22); and GSISRIST (SEQ ID NO:23);

5 a sequence of CDR2 selected from ITSGGTT (SEQ ID NO:24); ISRSGAGT (SEQ ID NO:25); ITTGGST (SEQ ID NO:26); TNWSSGNT (SEQ ID NO:27); and ISTGGTT (SEQ ID NO:28); and

a sequence of CDR3 selected from NTVKVVGGRLDNP DY (SEQ ID NO:29); VARPTKVDRDYATRREMYNY (SEQ ID NO:30); NSVAVVGGVIKSPDY (SEQ ID NO:31); AARKLDVPSRYSQHYDY (SEQ ID NO:32); and AAGWKVVRGSLEYEY (SEQ ID NO:33),

10

wherein the antibody or fragment thereof is specific for TcdB. The isolated or purified antibody or fragment thereof as just described may be selected from the group consisting of:

an isolated or purified antibody or fragment thereof comprising a sequence of CDR 1 of GNIFSINT (SEQ ID NO:19); CDR2 of ITSGGTT (SEQ ID NO:24); and CDR3 of NTVKVVGGRLDNP DY (SEQ ID NO:29);

15

an isolated or purified antibody or fragment thereof comprising a sequence of CDR 1 of GRTASGYG (SEQ ID NO:20); CDR2 of ISRSGAGT (SEQ ID NO:25); and CDR3 of VARPTKVDRDYATRREMYNY (SEQ ID NO:30);

an isolated or purified antibody or fragment thereof comprising a sequence of CDR 1 of GRTFSSGV (SEQ ID NO:21); CDR2 of ITTGGST (SEQ ID NO:26); and CDR3 of NSVAVVGGVIKSPDY (SEQ ID NO:31);

20

an isolated or purified antibody or fragment thereof comprising a sequence of CDR 1 of GLSRYA (SEQ ID NO:22); CDR2 of TNWSSGNT (SEQ ID NO:27); and CDR3 of AARKLDVPSRYSQHYDY (SEQ ID NO:32); or

an isolated or purified antibody or fragment thereof comprising a sequence of CDR 1 of GSISRIST (SEQ ID NO:23); CDR2 of ISTGGTT (SEQ ID NO:28); and CDR3 of AAGWKVVRGSLEYEY (SEQ ID NO:33).

25

The isolated or purified antibody or fragment thereof as described above may comprise a sequence selected from the group consisting of:

- QVQLVESGGGLVQPGGSLRLSCAASGNIFSINTMGWYRQAPGKQLELVAAITSGGTTSYT
DSVEGRFTISRDNNAKNAVYLMNSLKAEDTAVYYCNTVKVVGGRLDNPDYWGQGTQVTV
SS (SEQ ID NO:40);
- 5 QVKLEESGGGLVQPGGSLRLSCAASGRTASGYGMGWFRQAPGKEREVAAISRSGAGTL
NADFVKGRFTISRDNNAKNTVYLMNSLKPEDTAVYYCVARPTKVDRDYATRREMYNYWG
QGTQVTVSS (SEQ ID NO:41);
- QVKLEESGGGLVQAGGSLRLSCSASGRTFSSGVMGWFRQAPGKQRELVAAITTGGSTSY
TDSVKGRFTISRDNNAKNTVYLMNSLKPEDTAVYYCNSVAVVGGVIKSPDYWGQGTQVTV
SS (SEQ ID NO:42);
- 10 QVQLVESGGGSVQAGGSLRLSCAASGLSRYAMAWFRQGTGKEREVASTNWSSGNTPY
ADSVKGRFIISRDNNAKNTVYLMNSLKPEDTAIYYCAARKLDVPSRYSQHYDYWGQGTQV
TVSS (SEQ ID NO:43);
- QVQLVESGGDLVQAGGSLRLSCAASGSISRISTMGWYRQAPGKQRELVAITSTGGTTNYA
ESVKGRFTVSRDNNAKNTMYLMNSLKPEDTAVYYCAAGWKVVRGSLEYEYSGQGTQVTV
15 SS (SEQ ID NO:44);
- QVQLVESGGGLVQPGGSLRLSCAASGNIFSINTMGWYRQAPGKQLELVCAITSGGTTSYTDS
VEGRFTCSRDNNAKNAVYLMNSLKAEDTAVYYCNTVKVVGGRLDNPDYWGQGTQVTVSS
(SEQ ID NO:51), referred to herein as B5.2m;
- 20 QVKLEESGGGLVQPGGSLRLSCAASGRTASGYGMGWFRQAPGKEREVCAISRSGAGTLN
ADSVKGRFTCSRDNNAKNTVYLMNSLKPEDTAVYYCVARPTKVDRDYATRREMYNYWGQG
TQVTVSS (SEQ ID NO:52), referred to herein as B7.3m;
- QVKLEESGGGLVQAGGSLRLSCSASGRTFSSGVMGWFRQAPGKQRELVAITTGGSTSYTD
SVKGRFTCSRDNNAKNTVYLMNSLKPEDTAVYYCNSVAVVGGVIKSPDYWGQGTQVTVSS
(SEQ ID NO:53), referred to herein as B13.6m;
- 25 QVQLVESGGGSVQAGGSLRLSCAASGLSRYAMAWFRQGTGKEREVVCSTNWSSGNTPYAD
SVKGRFICSRDNNAKNTVYLMNSLKPEDTAIYYCAARKLDVPSRYSQHYDYWGQGTQVTVS
S (SEQ ID NO:54), referred to herein as B15.3m; and
- QVQLVESGGDLVQAGGSLRLSCAASGSISRISTMGWYRQAPGKQRELCATISTGGTTNYAES
VKGRFTCSRDNNAKNTMYLMNSLKPEDTAVYYCAAGWKVVRGSLEYEYSGQGTQVTVS
30 (SEQ ID NO:55), referred to herein as B15.5m,

or a sequence substantially identical thereto.

The isolated or purified antibody or fragment thereof as described above may be a single-domain antibody (sdAb); the sdAb may be of camelid origin.

5 The isolated or purified antibody or fragment thereof of as described herein may be in a multivalent display format.

The isolated or purified antibody or fragment thereof as described herein may be immobilized onto a surface.

10 The isolated or purified antibody or fragment thereof of the present invention may be linked to a cargo molecule; the cargo molecule may be a detectable agent, a therapeutic, a drug, a peptide, a protease, an enzyme, a carbohydrate moiety, or a cytotoxic agent; one or more liposomes loaded with a detectable agent, a therapeutic, a drug, a peptide, an enzyme, or a cytotoxic agent; or one or more nanoparticle, nanowire, nanotube, or quantum dots.

15 The present invention further encompasses a nucleic acid molecule encoding the isolated or purified antibody or fragment thereof as described above. The present invention also includes a vector comprising the nucleic acid molecule just described.

Also provided is a composition comprising one or more than one isolated or purified antibody or fragment thereof of the present invention and a pharmaceutically-acceptable carrier, diluent, or excipient.

20 The present invention further provides a method of treating a *Clostridium difficile* infection, comprising administering the isolated or purified antibody or fragment thereof of the present invention or the composition of described above to a subject in need thereof.

25 In another aspect, there is provided a method of capturing *Clostridium difficile* toxins, comprising contacting a sample with one or more than one isolated or purified antibody or fragment thereof of the present invention immobilized onto a surface, and allowing the toxin(s) to bind to the isolated or purified antibody or fragment thereof. The method just described may further comprise identifying the toxin by mass spectrometric methods and/or eluting the bound toxin.

30 The present invention additionally provides a method of detecting *Clostridium difficile* toxins, comprising contacting a sample with one or more than one isolated or purified antibody or fragment thereof linked to a cargo molecule, and detecting the bound antibody or fragment

thereof using a suitable imaging or detection technology. The cargo molecule may be a detectable agent.

The present invention provides isolated llama single-domain antibodies (V_HHs) capable of binding, detecting, capturing, and/or neutralizing *C. difficile* TcdA and TcdB. Without wishing to
5 be bound by theory, V_HHs targeting the toxin's receptor binding domain (RBD) may block the toxin-receptor interaction, thereby preventing toxin entry into the host cell; a critical initial step in the TcdA/B mechanism of action (Jank and Aktories, 2008). To do so, a hyperimmunized llama V_HH phage display library was constructed and panned with recombinant RBD fragments. The isolated V_HHs were then characterized for their ability to bind native toxins and
10 recombinant RBD fragments and the nature and relative positioning of epitopes. In addition, the ability of V_HHs to neutralize toxins in an *in vitro* cell cytotoxicity assay was assessed.

Several TcdA-specific V_HHs capable of neutralizing TcdA *in vitro* through high-affinity interactions with TcdA-RBD were found. V_HHs are extremely stable antigen-binding domains that are expressed at high-yields in recombinant organisms and are capable of neutralizing
15 infectious disease-related targets (Wesolowski et al, 2009). With respect to CDAD therapy, V_HHs could be administered systemically to target TcdA and TcdB as they share high sequence homology with human V_H domains, thus are well-tolerated in humans (Vu et al, 1997). Enhanced toxin neutralizing efficacy may be obtained by increasing their blood circulation half lives, size and avidity using various techniques, including chimeric formats of
20 anti-TcdA V_HHs linked to an Fc domain, generation of bi- or tri-specific antibody fusions with two or three anti-TcdA V_HHs recognizing unique epitopes, PEGylation, fusion to serum albumin, or fusion to serum albumin-specific antibody fragments. By targeting *C. difficile* virulence factors such as TcdA/B, selection pressure is removed from the organism, decreasing the chance of antibiotic resistance. A mutation in the RBD, which is conserved
25 among *C. difficile* isolates including hypervirulent 027 ribotype strains, is unlikely to benefit the organism and in the event it does occur, the toxin may lose its ability to enter host cells. As such, anti-TcdA/B V_HHs are logical agents to explore for CDAD therapy.

In order to improve the V_HHs' biophysical properties, the *C. difficile* TcdA-specific V_HHs were engineered to insert a non-canonical disulfide bond by introducing Ala/Gly⁵⁴→Cys⁵⁴ and
30 Ile⁷⁸→Cys⁷⁸ mutations, allowing for the formation of a second, non-native disulfide bond between FR2 and FR3 in the V_HH hydrophobic core. Disulfide bond formation was confirmed using a combination of proteolytic and chemical digestion coupled with MS² to precisely identify V_HH peptide fragments harboring the introduced disulfide bond. The mutant antibodies were compared to their wild-type counterparts with respect to yield, solubility, affinity for TcdA,
35 thermal unfolding at neutral and acidic pH and protease resistance. Mutant V_HHs were found

to be soluble, non-aggregating monomers, possessing similar affinity constants to that of WT V_H Hs. SPR binding experiments revealed most mutant V_H Hs possessed 1- to 5-fold weaker affinity constants relative to wild-type, which is consistent with other reports in the art.

CD spectroscopy was used to compare wild-type and mutant V_H H secondary structure, tertiary
5 structure, thermal stability (T_m and T_{onset}), and thermal refolding efficiency (TRE). Comparisons of V_H H secondary and tertiary structure with far-UV and near-UV CD spectroscopy strongly suggested structural differences between wild-type and mutants, at both neutral and acidic pH. For all mutants, peak intensity and selective peak minima shifts were observed, although the overall spectral profiles remained very similar in all wild type/mutant pairs. More specifically,
10 mutants consistently showed rightward peak shifts in the peak range of 230 nm – 235 nm (far-UV CD spectra) and around 297 nm (near-UV CD spectra) compared to wild type V_H Hs. Such patterns may be used as signatures that could be used to quickly identify V_H Hs containing a properly formed non-canonical disulfide bond, as could SDS-PAGE motility values since, compared to wild type V_H Hs, mutants consistently moved slower in SDS-PAGE gels. Thus, the
15 far- and near-UV CD spectral data suggests the introduced disulfide bond changes the structure of V_H Hs. This is consistent with the observed perturbations in affinities and specificities and increased GI protease resistance of the mutant V_H Hs compared to the wild types.

CD spectroscopy thermal denaturation experiments were performed to show a profound and
20 significant ($p < 0.05$) increase in the T_m s and T_{onset} s of mutant V_H Hs at both neutral and acidic pH. Increases in midpoint temperature of unfolding (T_m) ranging from ~4 to ~12°C were observed for all mutants, at both neutral and acidic pH. Without wishing to be bound by theory, for the mutant V_H Hs with a higher thermostability gain, the non-canonical disulfide linkage may have been a better fit to overall fold. For example, A19.2m and A24.1m showed the lowest
25 thermostability gains and this would explain why they were transformed into non-specific binders upon mutation. For A4.2m on the other hand, the non-canonical disulfide linkage seems to be a natural fit, as it increased its T_m the most (by almost 12°C) and significantly improved GI protease resistance (with the highest increase in pepsin resistance), all without adversely affecting the K_D .

30 Digestion of the V_H Hs with major gastrointestinal proteases at biologically relevant concentrations revealed a significant ($p < 0.05$) increase in pepsin resistance for all mutants; however, increases in resistance profiles to chymotrypsin and trypsin were not as universal. Each wild-type and mutant V_H H pair possessed an identical number of theoretical protease cleavage sites (data not shown); thus it seems that the added disulfide bond leads to a more
35 compact and thermodynamically stable V_H H structure, preventing pepsin and chymotrypsin

from accessing proteolytic cleavage sites. V_HH refolding was also examined using CD spectroscopy. While wild-type refolding was better than mutant V_HH refolding at neutral pH the reverse was true under stringent conditions (acidic pH). At acidic pH, 5 of 6 mutant V_HHs possessed greater refolding efficiency than wild-type after complete thermal denaturation with the majority essentially showing reversible thermal unfolding.

The introduction of the Cys54/Cys78 disulfide linkage into V_HHs led to increases in both T_m and thermodynamic stability. Proteins with higher T_m are also less likely to unfold [62]. These may be the reasons why the present mutants were more resistant to acid-induced unfolding at 37°C, supported by the higher T_{onset} s and pepsin resistance of the mutant V_HHs. This benefit is not realized for mutants against trypsin, possibly because their cleavage sites are at hydrophilic residues (Lys or Arg), which may be in more exposed regions of the V_HH, possibly located in the CDR regions. Further, these regions would not be protected by stabilizing the core of the structure. An increase in T_{onset} temperatures for mutants at the physiological condition representative of the stomach (pH 2.0 and 37°C) to values significantly above 37°C (T_{onset} s from 45°C – 53°C) was observed. This indicates that the mutants should remain fully folded at 37°C in the stomach, hence resisting pepsin degradation (and denaturation) to a higher extent than wild type V_HHs, which is supported by the pepsin digestion experiments herein.

The toxin A neutralizing efficacy of the disulfide bond mutant V_HHs was 3 — 4 fold weaker compared to the wild-type V_HHs in toxin A neutralization in cell-based assays, presumably a reflection in the reduced affinities of 3 of 4 V_HHs for the toxin. Under stringent conditions *in vivo*, the lower affinity mutants may actually be more efficacious than the higher affinity wild-type V_HHs due to their greater stability.

It is presently shown that the introduction of a second disulfide bond into the hydrophobic core of a panel of llama V_HHs increased thermal stability and GI protease resistance; the approach is both effective and general. While affinity, specificity, and expression yield may be reduced, the mutants comprising additional disulfide bond outperformed the wild-type V_HHs under more stringent physiological conditions; this far outweighs the reductions in affinity.

Protein-based oral therapeutics have several conceived advantages over systemic administration: convenience, patient compliance, lower cost, pain-free administration, drug purity, flexibility in production source (i.e., bacterial, plant, etc.), and fewer concerns over immunogenicity. Despite the many advantages of orally administering protein therapeutics, few successes have been realized due to the destabilizing environment of the GI tract. Of the major GI proteases, pepsin is considered the primary cause of antibody degradation and

hence a major obstacle facing orally-delivered antibody therapeutics. The introduction of an additional disulfide bond in the hydrophobic core of the anti-TcdA V_HHs not only increased thermal stability at neutral pH, but also represents a generic strategy to increase antibody stability at low pH and impart pepsin resistance which is desirable for protein-based oral
 5 therapeutics.

Additional aspects and advantages of the present invention will be apparent in view of the following description. The detailed description and examples, while indicating preferred embodiments of the invention, are given by way of illustration only, as various changes and
 10 modifications within the scope of the invention will become apparent to those skilled in the art in light of the teachings of this invention.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features of the invention will now be described by way of example, with reference to the appended drawings, wherein:

15 FIGURE 1 relates to the isolation of anti-TcdA/B V_HHs. FIGURE 1A is a schematic representation of native TcdA/B and recombinant fragments of the cell receptor-binding domain (TcdA-RBD-f1 and TcdB-RBD-f1) used for llama immunization and library panning. Numbers represent the amino acid residues of each toxin, labelled from the N-termini (1) to C-termini (2710/2366 for TcdA and TcdB, respectively). Drawings are not to scale. GT =
 20 glucosyltransferase domain; CP = cysteine protease domain; HR = hydrophobic membrane insertion domain; RBD = cell-receptor binding domain. FIGURE 1B is an SDS-PAGE profile of the purified *C. difficile* toxins (3 µg per lane; from *C. difficile* strain 10463) used herein. The upper arrow shows full-length TcdA (308 kDa) and TcdB (269 kDa). FIGURE 1C shows ELISA results demonstrating a total llama serum response for the recombinant RBD fragments.
 25 Serum was prepared from llama blood drawn 57 days after the initial immunization. Immune (A): immune serum against TcdA-RBD-f1; Immune (B): immune serum against TcdB-RBD-f1; Pre-Immune: pre-immune serum against TcdA-RBD-f1. FIGURE 1D shows ELISA results demonstrating the llama heavy-chain IgG (HCAb) G1 fraction response was specific for the recombinant RBD fragments. Serum was fractionated from llama blood drawn 84 days after
 30 the initial immunization and the G1 fraction shown did not recognize PEB3 or CPS, two unrelated antigens. FIGURE 1E shows the SDS-PAGE profile of purified V_HHs (2 µg per lane) isolated from the hyperimmunized llama phage display library. Molecular weight markers (M) are given in kDa. NR: non-reducing.

FIGURE 2 is an amino acid sequence alignment of the anti-TcdA/B V_HHs isolated. Framework regions, FRs, and complementarity-determining regions, CDRs (shaded in grey), are grouped according to the IMGT numbering system. Hallmark positions, 42, 49, 50 and 52, where V_HHs can be distinguished from V_H based on amino acid identity are illustrated with asterisks. V_Hs generally have V, G, L and W at positions 42, 49, 50 and 52, respectively; in many V_HHs, the
5 aforementioned residues are replaced with F/Y, E/Q/R, R and F/L, as shown.

FIGURE 3 describes the construction, expression and functional characterization of pentameric versions of monomeric anti-TcdA V_HHs. FIGURE 3A is a schematic diagram of a V_HH pentamer. V_HH monomers expressed as fusions to the verotoxin B (VTB) subunit self-
10 assembly into pentameric structures in *E. coli*. FIGURE 3B shows an SDS-PAGE gel containing eluted IMAC fractions of purified V_HH pentamers A5.1p and A20.1p. Molecular weight markers (M) are given in kDa. NR: non-reducing. Arrows indicate the location of purified VTB-V_HH monomers. FIGURE 3C shows ELISA results comparing the functional activity of V_HH monomers (A5.1 and A20.1) and V_HH pentamers (A5.1p and A20.1p) to wells coated with
15 TcdA. Equimolar concentrations of monomer/pentamer were used in the assay relative to the V_HH monomer. Monomer/pentamer binding was detected with rabbit anti-His₆ IgG-HRP and absorbances were read at 450 nm.

FIGURE 4 depicts the toxin binding characteristics of V_HHs. ELISA demonstrating the anti-TcdA V_HHs (FIGURE 4A) and anti-TcdB V_HHs (FIGURE 4B) recognize native toxins and
20 recombinant RBD fragments. Wells were coated with molar equivalent concentrations. FIGURE 4C and FIGURE 4D shows binding of various concentrations of V_HHs to immobilized TcdA or TcdB, respectively.

FIGURE 5 shows the gel filtration profiles of anti-TcdA V_HHs obtained from a Superdex™ 75 column. The single peak for all WT V_HHs suggests all are non-aggregating monomeric
25 species.

FIGURE 6 shows surface plasmon resonance analysis of anti-TcdA/B V_HHs. Sensorgrams of TcdA-specific V_HHs A4.2, A5.1, A19.2, A20.1, A24.1 and A26.8 binding to immobilized TcdA (FIGURES 6A-F) and TcdB-RBD-f1 (Figure 1) binding to immobilized TcdB-specific V_HHs B5.2, B13.6 and B15.5 (FIGURES 6G-I) are shown. In experiments involving TcdA-specific
30 V_HHs, TcdA was immobilized on CM5-dextran chips and monomeric V_HHs were passed over at concentration ranges noted on each sensorgram, giving affinity constants ranging from 2 nM to 290 nM. In experiments involving TcdB-specific V_HHs, antibodies were immobilized on CM5-dextran chips and TcdB-RBD-f1 ranging in concentration from 2 μM to 200 nM was passed

over, giving affinity constants ranging from 100 nM to 400 nM. Black lines represent raw data measurements and grey lines represent fitted curves. All data presented here showed acceptable fitting to a 1:1 binding model. Rate and affinity constants are shown in Table 1.

FIGURE 7 shows a subset of TcdB-specific V_HHs with complex binding to recombinant TcdB-RBD. Surface plasmon resonance sensorgrams for V_HHs that showed binding to TcdB-RBD-f1, but whose data was non analyzable. The recombinant TcdB-RBD-f1 fragment (2 μM → 200 nM) was passed over immobilized V_HHs (526 - 1209 RUs). (A) B7.3, (B) B13.2, (C) B13.3, and (D) B15.3.

FIGURE 8 shows the *in vitro* neutralization of TcdA cytotoxicity with anti-TcdA V_HHs. Dose-response curves of TcdA (FIGURE 8A) and TcdB (FIGURE 8B) on monolayers of IMR-90 human lung fibroblast (HLF) cells. The percentage of cell rounding was scored from 0% to 100% of the cells affected. FIGURES 8C-E show neutralization of TcdA-induced cell rounding with V_HHs at 24 h post addition of 100 ng/ml TcdA. The final concentration of V_HHs in each assay well were 1000 nM (FIGURE 8C), 10 nM (FIGURE 8D) and 0.1 nM (FIGURE 8E) and V_HHs were added as singles, pairs, or triplet combinations. White bars represent single V_HHs or PBS control, grey bars represent paired combinations and black bars represent triplet combinations. Combinations of V_HHs (i.e., pairs and triples) increased toxin neutralizing efficacy. FIGURE 8F shows representative photographs of TcdA neutralization with 1000 nM V_HH were taken 24 h post toxin addition. The black bar represents 100 μm.

FIGURE 9 shows results of epitope typing experiments. Anti-toxin V_HHs recognize conformational (A4.2, A5.1, A20.1, A26.8) and linear (A19.2) epitopes on native *C. difficile* toxin and recombinant fragments of the cell receptor-binding domain. FIGURE 9A shows ELISA results on TcdA treated with various temperatures for 30 min before probing with V_HHs. At treatment temperatures above the TcdA midpoint unfolding temperature (T_m), binding of 4 out of 5 TcdA-specific V_HHs was abolished. The dotted line represents the TcdA T_m of ~55°C. FIGURE 9B shows Western blots (reducing/denaturing) probed with His-tagged anti-TcdA V_HHs or control mouse anti-TcdA IgG (PCG-4). Binding was detected with nickel-AP or goat anti-mouse IgG-AP, respectively. Of the V_HHs tested, only A19.2 recognized denatured TcdA and the secondary conjugates did not bind denatured TcdA. FIGURE 9C shows Western blots (native-PAGE) probed with anti-TcdA V_HHs or control PCG4. V_HHs and PCG4 bound TcdA. The goat anti-mouse IgG-AP conjugates strongly cross-reacted with TcdA in the absence of PCG4.

FIGURE 10 shows results of Biacore™ co-injection experiments, which were used to determine if pairs of V_HHs could bind TcdA simultaneously. The Biacore™ sensorgrams of all

paired combinations of A4.2, A5.1, A20.1 and A26.8, in both orientations, are shown. Dashed lines represent injection of a single V_HH followed by injection of buffer. Solid lines represent co-injections of the first V_HH followed by injection of a second V_HH . For all experiments, 80 μ l of each V_HH at a concentration 20 x its K_D was injected over 10,287 RUs of immobilized TcdA at 40 μ l/min. In general, A4.2, A5.1 and A26.8 appeared to share an overlapping epitope as no significant increase in response was found upon injection of the second species. Conversely, A20.1 appeared to bind a distinct, non-overlapping epitope.

FIGURE 11 shows Biacore results indicating that a subset of V_HH s bind TcdA at overlapping epitopes. The three V_HH s suspected of sharing an overlapping epitope (Figure 7) were injected alone (A4.2, A5.1, or A26.8) and as a triplet mixture ("Mix") over immobilized TcdA. The Biacore sensorgram illustrates similar R_{max} (~160-200 RUs) values for individual V_HH s with no increase in response upon injection of the mixed population, indicating these antibodies recognize an overlapping epitope on TcdA. If the mixture of V_HH s were free to bind at non-overlapping sites, one would expect an R_{max} value for the mixtures to reach the sum of all individual R_{max} values (i.e., ~540 RUs). In all experiments, 80 μ l of V_HH s were injected at 40 μ l/min and used at 20 x their K_D concentrations.

FIGURE 12 shows results of Biacore analysis, which revealed that two trisaccharides, CD-grease (CD) and Le^X -AmHex (Le^X), known to interact at the carbohydrate binding sites of TcdA-RBD, did not inhibit V_HH binding to immobilized TcdA. All four neutralizing V_HH s were tested and one representative example is shown. FIGURE 12A shows the responses of A26.8 binding TcdA, CD-grease binding TcdA and co-injection of A26.8 and CD-grease. FIGURE 12B shows the responses of A26.8 binding TcdA, Le^X -AmHex binding TcdA and co-injection of A26.8 and Le^X -AmHex. FIGURES 12C and 12D show subtraction of the response generated from either trisaccharide binding to TcdA from co-injection experiments reveals a near identical response to that of A26.8 alone, an indication that V_HH binding to TcdA is not inhibited by the trisaccharides. In all experiments, V_HH s were used at their K_D concentrations and trisaccharides at their apparent K_D (2 mM).

FIGURE 13 shows Biacore results indicating V_HH binding to TcdA does not impair binding of the trisaccharide CD-grease (CD), which is known to bind TcdA-RBD. FIGURE 13A shows the injection of A20.1 (A, grey and dashed lines), followed by injection of A20.1 (B, dashed line) or A20.1 + CD-grease (B, grey line), and finally injection of Biacore buffer (C, grey and dashed lines). FIGURE 13B shows the injection of A26.8 (A, grey and dashed lines), followed by injection of A26.8 (B, dashed line) or co-injection of A26.8 + CD-grease (B, grey line), and finally injection of Biacore buffer (C, grey and dashed lines). These results suggest V_HH binding is not at or in the carbohydrate binding site on TcdA-RBD.

FIGURE 14 shows the alignment and comparison of wild-type and mutant V_HH amino acid sequences. Wild-type V_HH sequences are shown with a single disulfide bond between Cys²³ and Cys¹⁰⁴. A second disulfide bond was introduced through mutation of Ala⁵⁴/Gly⁵⁴ and Ile⁷⁸ to Cys⁵⁴ and Cys⁷⁸ in framework region (FR) 2 and FR3, respectively. Disulfide bonds are shown as black lines. Bolded residues illustrate the disulfide bond-linked peptides identified by nanoRPLC-ESI-MS analysis on CNBr and trypsin digested mutant V_HHs. Amino acid numbering and CDR designation is based on the IMGT system.

FIGURE 15A shows a non-reducing (NR) SDS-PAGE analysis and Western blot (WB) probed with an anti-His₆ IgG on IMAC-purified mutant V_HHs. M: molecular weight marker in kDa. FIGURE 15B is a representative SDS-PAGE analysis showing mutant V_HHs run slower than the corresponding wild-type V_HHs under non-reducing conditions.

FIGURE 16 confirms the disulfide bond formation between residues Cys⁵⁴ and Cys⁷⁸ by MS². FIGURE 16A shows a SDS-PAGE gel under non-reducing (NR) conditions of V_HHs (3 µg per lane), which illustrates near-complete digestion with CNBr and trypsin. Untreated A5.1m was added as a control (Ctl). M: molecular weight marker in kDa. FIGURE 16B shows a MaxEnt 3 deconvoluted CID-MS² spectrum of the m/z 526.25 (3+) ion of the disulfide-linked peptide EFVCVITR (P1) – FTCSR (P2), encompassing the Cys⁵⁴-Cys⁷⁸ disulfide bond, from CNBr/trypsin digested A5.1m. Amino acid positions are based on the IMGT numbering system.

FIGURE 17 shows the characterization of mutant anti-TcdA V_HHs which possessed a second disulfide bond in the hydrophobic core that were introduced by mutation of two amino acids to cysteine. FIGURE 17A shows a comparison of the Size exclusion chromatography (SEC) analysis of wild-type (WT) anti-TcdA V_HHs (top) and mutant anti-TcdA V_HHs (bottom) obtained from a SuperdexTM 75 column. Similar size exclusion profiles were obtained for mutant and wild-type, indicating the second disulfide bond does not promote the formation of interdomain disulfide-bonds or multimeric mutant V_HHs. The elution volumes (V_es) of SEC molecular weight standards are shown with arrows and are aligned relative to the A4.2 and A4.2m chromatograms. a: ovalbumin (MW = 43.0 kDa, V_e = 8.90 ml); b: carbonic anhydrase (MW = 30.0 kDa, V_e = 9.71 ml); c: trypsin inhibitor (MW = 20.1 kDa, V_e = 11.06 ml); d: α-lactalbumin (MW = 14.4 kDa, V_e = 11.97 ml); e: vitamin B (MW = 1.3 kDa, V_e = 18.7 ml). The equation of the line of a standard curve generated from these standards was LOG₁₀MW = -0.1539V_e + 2.9949 (r² = 0.9995). From this equation the V_HH apparent MWs ranged from 9.8 – 13.6 kDa, indicating monomeric V_HHs. FIGURE 17B shows surface plasmon resonance (SPR) sensorgrams for four mutant V_HHs binding to immobilized TcdA. Grey lines represent raw data measurements and black lines represent fitted curves. Kinetic and affinity constants are given in Table 4. Binding of A19.2m and A24.1m to TcdA was non-specific, and the kinetic and

affinity constants could not be determined. The binding shows mutant V_HHs with a second disulfide bond retain high-affinity binding to TcdA.

FIGURE 18 shows far-UV CD spectra of V_HHs at neutral and acidic pH. CD scans (210 nm – 260 nm) were performed at 25°C on V_HHs (50 µg/mL) equilibrated for 2 h in 10 mM phosphate buffer (pH 7.3) or 10 mM phosphate buffer + 50 mM HCl (pH 2.0). The spectra represent the mean residue ellipticity of 8 data accumulations collected from 2 independent experiments. Black lines: wild-type V_HH; grey lines: mutant V_HH.

FIGURE 19 shows near-UV CD analysis of V_HHs at neutral and acidic pH. CD scans (250 nm – 340 nm) were performed at 25°C on V_HHs (250 µg/mL) equilibrated for 2 h in 10 mM phosphate buffer (pH 7.3) or 10 mM phosphate buffer + 50 mM HCl (pH 2.0). The spectra represent the mean residue ellipticity from 8 data accumulations collected from 2 independent experiments. Black lines: wild-type V_HH; grey lines: mutant V_HH.

FIGURE 20 shows far-UV CD scans used to determine V_HH refolding efficiencies under neutral and acidic pH conditions. A5.1 is shown as a representative example. FIGURE 20A shows CD spectra collected on equilibrated V_HHs (50 µg/ml) at 25°C before heat treatment (scan 1, *solid lines*), after exposure to 96°C for 20 min (scan 2, *dotted lines*), and after cooling to 25°C for 3 h (scan 3, *dashed lines*). Scans are an average of 4 data accumulations. FIGURE 20B shows a summary of thermal refolding efficiencies at pH 7.3 and pH 2.0, calculated using Equation 2 and following the changes in ellipticity at 215 nm. Dots represent the mean thermal refolding efficiency (TRE) of individual V_HHs from two independent experiments with 4 data accumulations in each experiment. Bars represent the mean TRE of each group of V_HHs.

FIGURE 21 shows results of circular dichroism analysis for the determination of WT and mutant V_HH melting temperatures at neutral and acidic pH. FIGURE 21A shows unfolding transition curves for all six WT and mutant anti-TcdA V_HHs at neutral pH (7.3) and acidic pH (2). V_HH thermal unfolding midpoint temperatures (T_m s) were determined using CD spectroscopy by following antibody unfolding (50 µg/mL) at 215 nm in 10 mM phosphate buffer +/- 50 mM HCl. T_m was determined for each curve by Boltzmann non-linear curve fitting analysis in GraphPad Prism. The goodness of curve fit (r^2) ranged from 0.9901 – 0.9995. In the case of V_HHs with few lower baseline data points the T_m is a minimal estimate.. FIGURES 21B and 21C show a summary of the V_HH T_m s and T_{onset} s, respectively. Each dot represents individual V_HH and the bar represents the mean T_m or T_{onset} value in each group. P-values were determined using the unpaired two-tailed *t*-test. The T_m values are summarized in Table 6.

FIGURE 22 shows the resistance profiles of WT and mutant V_HHs to the major gastrointestinal proteases pepsin, trypsin and chymotrypsin. FIGURE 22A is a representative SDS-PAGE gel comparing the profiles of WT and mutant A5.1 V_HH after no treatment or digestion with various ratios of pepsin for 1 h at 37°C. V_HH epitope tags ("tag"), consisting of c-Myc and His₆, were preferentially cleaved by all proteases (confirmed by mass spectrometry analysis – data not shown). Densitometric analysis of SDS-PAGE gels allowed for the determination of a percent of retained V_HH structure, which was denoted percent resistance. FIGURE 22B-D summarizes the percent resistance of WT and mutant V_HHs to pepsin, trypsin, and chymotrypsin after digestion for 1 h at 37°C using a protease concentration of 100 µg/ml (1:2.4 ratio of protease:V_HH). Error bars represent the SEM obtained from 3 independent digestions for each V_HH. FIGURE 22E shows a summary of the V_HH resistance to each protease. Dots represent the mean (n = 3) protease resistance profile of each V_HH relative to undigested controls and the black bars represent the median resistance of each group. P-values were determined using the unpaired two-tailed Mann-Whitney *U* test. WT: wild-type V_HH; Mut: mutant V_HH; Chymo: chymotrypsin. The percent V_HH resistance to each protease is given in Table 8. In A, 1:240 and 1:24 ratios correspond to pepsin concentrations of 1 µg/ml and 10 µg/ml, respectively, in reaction mixtures.

FIGURE 23 shows the correlation between V_HH resistance to pepsin and thermal stability at pH 2. FIGURE 23A is a graph showing linear regression between V_HH pepsin resistance and V_HH *T_m* at pH 2.0. Boxes show the wild-type (WT) and mutant (Mut) V_HHs, respectively. Linear regression analysis gave a correlation coefficient of $r^2 = 0.735$ and a significantly non-zero slope of the line ($p = 0.0004$). FIGURE 23B is a graph showing linear regression between wild-type V_HH pepsin resistance and wild-type V_HH *T_{onset}* at pH 2.0. The *T_{onset}* is defined as the temperature at which 5% of the V_HH is unfolded. Linear regression analysis gave a correlation coefficient of $r^2 = 0.975$ and a significantly non-zero slope of the line ($p = 0.0002$). FIGURE 23C shows SPR analysis (*bottom*) on mutant V_HHs digested with pepsin (100 µg/ml, 1 h, 37°C). The pepsin-treated V_HHs retained their ability to bind surface-immobilized TcdA. SDS-PAGE gel (*top*) shows untreated (lanes 1, 3, 5, 7) and pepsin-digested (lanes 2, 4, 6, 8) V_HHs used for SPR. The contents of lanes 1 thru 8 are listed in the box. Normalized *k_{off}*s for pepsin treated V_HHs were similar to the *k_{off}* of untreated controls (*box* and Table 2). M: molecular weight markers in kDa; WT: wild-type V_HH; Mut: mutant V_HH; P: pepsin; R: reducing SDS-PAGE conditions.

FIGURE 24 is a bar graph showing that mutant V_HHs retain TcdA-neutralizing capacity. Confluent monolayers of IMR-90 human lung fibroblasts were incubated with TcdA or TcdA + V_HHs for 24 h, and the percentage of cells rounded was scored. V_HHs (wild-type (WT) or

mutant (Mut)) were added as pooled mixtures of A4.2, A5.1, A20.1, and A26.8 (250 nM each) or A4.2m, A5.1m, A20.1m, and A26.8m (250 nM each).

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to *Clostridium difficile*-specific antibodies and uses thereof. More specifically, the present invention relates to *Clostridium difficile* toxin-specific antibodies and uses thereof.

The present invention provides isolated llama single-domain antibodies (V_HHs) capable of binding and neutralizing *C. difficile* TcdA and TcdB. Without wishing to be bound by theory, V_HHs targeting the toxin's receptor binding domain (RBD) may block the toxin-receptor interaction, thereby preventing toxin entry into the host cell; this represents a critical initial step in the TcdA/B mechanism of action (Jank and Aktories, 2008).

Thus, the present invention provides an isolated or purified antibody or fragment thereof, comprising

a sequence of complementarity determining region (CDR) 1 selected from GRTFNTLS (SEQ ID NO:1); GRTFSMYR (SEQ ID NO:2); GRTLSSYI (SEQ ID NO:3); GRTFSMDP (SEQ ID NO:4); IRSFSNRN (SEQ ID NO:5); and ERTFSRYP (SEQ ID NO:6);

a sequence of CDR2 selected from VSRSGGST (SEQ ID NO:7); ITRNGSST (SEQ ID NO:8); ISRRGGNS (SEQ ID NO:9); GSSTGRTT (SEQ ID NO:10); ISWGGGST (SEQ ID NO:11); and ISSTGTST (SEQ ID NO:12); and

a sequence of CDR3 selected from AAAATKSNTTAYRLSFDY (SEQ ID NO:13); AATSGSSYLDAAHVYDY (SEQ ID NO:14); AADGSVAGWGRRSVSVSSYDY (SEQ ID NO:15); AAAPYGANWYRDEYAY (SEQ ID NO:16); AAIEFGHNIATSSDEYDY (SEQ ID NO:17); and AVNSQRTRLQDPNEYDY (SEQ ID NO:18),

wherein the antibody or fragment thereof is specific for TcdA. The isolated or purified antibody or fragment thereof as just described may comprise

a sequence of CDR 1 of GRTFNTLS (SEQ ID NO:1); CDR2 of VSRSGGST (SEQ ID NO:7); and CDR3 of AAAATKSNTTAYRLSFDY (SEQ ID NO:13);

a sequence of CDR 1 of GRTFSMYR (SEQ ID NO:2); CDR2 of ITRNGSST (SEQ ID NO:8); and CDR3 of AATSGSSYLDAAHVYDY (SEQ ID NO:14);

a sequence of CDR 1 of GRTLSSYI (SEQ ID NO:3); CDR2 of ISRRGGNS (SEQ ID NO:9); and CDR3 of AADGSVAGWGRRSVSVSSYDY (SEQ ID NO:15);

a sequence of CDR 1 of GRTFSMDP (SEQ ID NO:4); CDR2 of GSSTGRIT (SEQ ID NO:10); and CDR3 of AAAPYGANWYRDEYAY (SEQ ID NO:16);

5 a sequence of CDR 1 of IRSFSNRN (SEQ ID NO:5); CDR2 of ISWGGGST (SEQ ID NO:11); and CDR3 of AAEFGHNIATSSDEYDY (SEQ ID NO:17); or

a sequence of CDR 1 of ERTFSRYP (SEQ ID NO:6); CDR2 of ISSTGTST (SEQ ID NO:12); and CDR3 of AVNSQRTLQDPNEYDY (SEQ ID NO:18).

The present invention also provides an isolated or purified antibody or fragment thereof,
10 comprising

a sequence of complementarity determining region (CDR) 1 selected from GNIFSINT (SEQ ID NO:19); GRTASGYG (SEQ ID NO:20); GRTFSSGV (SEQ ID NO:21); GLSRYA (SEQ ID NO:22); and GSISRIST (SEQ ID NO:23);

15 a sequence of CDR2 selected from ITSGGTT (SEQ ID NO:24); ISRSGAGT (SEQ ID NO:25); ITTGGST (SEQ ID NO:26); TNWSSGNT (SEQ ID NO:27); and ISTGGTT (SEQ ID NO:28); and

a sequence of CDR3 selected from NTVKVVGGRLDNP DY (SEQ ID NO:29); VARPTKVD RDYATRREMYNY (SEQ ID NO:30); NSVAVVGGVIKSPDY (SEQ ID NO:31); AARKLDVPSRYSQHYDY (SEQ ID NO:32); and AAGWKVVRGSLEYEY (SEQ
20 ID NO:33),

wherein the antibody or fragment thereof is specific for TcdB. The isolated or purified antibody or fragment thereof as just described may comprise

a sequence of CDR 1 of GNIFSINT (SEQ ID NO:19); CDR2 of ITSGGTT (SEQ ID NO:24); and CDR3 of NTVKVVGGRLDNP DY (SEQ ID NO:29);

25 a sequence of CDR 1 of GRTASGYG (SEQ ID NO:20); CDR2 of ISRSGAGT (SEQ ID NO:25); and CDR3 of VARPTKVD RDYATRREMYNY (SEQ ID NO:30);

a sequence of CDR 1 of GRTFSSGV (SEQ ID NO:21); CDR2 of ITTGGST (SEQ ID NO:26); and CDR3 of NSVAVVGGVIKSPDY (SEQ ID NO:31);

a sequence of CDR 1 of GLSRYA (SEQ ID NO:22); CDR2 of TNWSSGNT (SEQ ID NO:27); and CDR3 of AARKLDVPSRYSQHYDY (SEQ ID NO:32); or

a sequence of CDR 1 of GSISRIST (SEQ ID NO:23); CDR2 of ISTGGTT (SEQ ID NO:28); and CDR3 of AAGWKVVRGSLEYEY (SEQ ID NO:33).

- 5 The term "antibody", also referred to in the art as "immunoglobulin" (Ig), used herein refers to a protein constructed from paired heavy and light polypeptide chains; various Ig isotypes exist, including IgA, IgD, IgE, IgG, and IgM. When an antibody is correctly folded, each chain folds into a number of distinct globular domains joined by more linear polypeptide sequences. For example, the immunoglobulin light chain folds into a variable (V_L) and a constant (C_L) domain, while the heavy chain folds into a variable (V_H) and three constant (C_H , C_{H2} , C_{H3}) domains. Interaction of the heavy and light chain variable domains (V_H and V_L) results in the formation of an antigen binding region (Fv). Each domain has a well-established structure familiar to those of skill in the art.

- The light and heavy chain variable regions are responsible for binding the target antigen and can therefore show significant sequence diversity between antibodies. The constant regions show less sequence diversity, and are responsible for binding a number of natural proteins to elicit important biochemical events. The variable region of an antibody contains the antigen binding determinants of the molecule, and thus determines the specificity of an antibody for its target antigen. The majority of sequence variability occurs in six hypervariable regions, three each per variable heavy (V_H) and light (V_L) chain; the hypervariable regions combine to form the antigen-binding site, and contribute to binding and recognition of an antigenic determinant. The specificity and affinity of an antibody for its antigen is determined by the structure of the hypervariable regions, as well as their size, shape and chemistry of the surface they present to the antigen. Various schemes exist for identification of the regions of hypervariability, the two most common being those of Kabat and of Chothia and Lesk. Kabat et al (1991) define the "complementarity-determining regions" (CDR) based on sequence variability at the antigen-binding regions of the V_H and V_L domains. Chothia and Lesk (1987) define the "hypervariable loops" (H or L) based on the location of the structural loop regions in the V_H and V_L domains. As these individual schemes define CDR and hypervariable loop regions that are adjacent or overlapping, those of skill in the antibody art often utilize the terms "CDR" and "hypervariable loop" interchangeably, and they may be so used herein. For this reason, the regions forming the antigen-binding site are presently referred to herein as CDR L1, CDR L2, CDR L3, CDR H1, CDR H2, CDR H3 in the case of antibodies comprising a V_H and a V_L domain; or as CDR1, CDR2, CDR3 in the case of the antigen-binding regions of either a heavy chain or a light chain.
- 35 The CDR/loops are referred to herein according to the IMGT numbering system (Lefranc, M.-

P. et al., 2003), which was developed to facilitate comparison of variable domains. In this system, conserved amino acids (such as Cys23, Trp41, Cys104, Phe/Trp118, and a hydrophobic residue at position 89) always have the same position. Additionally, a standardized delimitation of the framework regions (FR1: positions 1 to 26; FR2: 39 to 55; 5 FR3: 66 to 104; and FR4: 118 to 128) and of the CDR (CDR1: 27 to 38, CDR2: 56 to 65; and CDR3: 105 to 117) is provided.

An "antibody fragment" as referred to herein may include any suitable antigen-binding antibody fragment known in the art. The antibody fragment may be a naturally-occurring antibody fragment, or may be obtained by manipulation of a naturally-occurring antibody or by using 10 recombinant methods. For example, an antibody fragment may include, but is not limited to a Fv, single-chain Fv (scFv; a molecule consisting of V_L and V_H connected with a peptide linker), Fab, $F(ab')_2$, single domain antibody (sdAb; a fragment composed of a single V_L or V_H), and multivalent presentations of any of these.

In a non-limiting example, the antibody fragment may be an sdAb derived from naturally- 15 occurring sources. Heavy chain antibodies of camelid origin (Hamers-Casterman et al, 1993) lack light chains and thus their antigen binding sites consist of one domain, termed V_{HH} . sdAb have also been observed in shark and are termed V_{NAR} (Nuttall et al, 2003). Other sdAb may be engineered based on human Ig heavy and light chain sequences (Jespers et al, 2004; To et al, 2005). As used herein, the term "sdAb" includes those sdAb directly isolated from V_H , V_{HH} , 20 V_L , or V_{NAR} reservoir of any origin through phage display or other technologies, sdAb derived from the aforementioned sdAb, recombinantly produced sdAb, as well as those sdAb generated through further modification of such sdAb by humanization, affinity maturation, stabilization, solubilization, e.g., camelization, or other methods of antibody engineering. Also encompassed by the present invention are homologues, derivatives, or fragments that retain 25 the antigen-binding function and specificity of the sdAb.

A person of skill in the art would be well-acquainted with the structure of a single-domain antibody (see, for example, 3DWT, 2P42 in Protein Data Bank). An sdAb comprises a single immunoglobulin domain that retains the immunoglobulin fold; most notably, only three CDR/hypervariable loops form the antigen-binding site. However, and as would be understood 30 by those of skill in the art, not all CDR may be required for binding the antigen. For example, and without wishing to be limiting, one, two, or three of the CDR may contribute to binding and recognition of the antigen by the sdAb of the present invention. The CDR of the sdAb or variable domain are referred to herein as CDR1, CDR2, and CDR3, and numbered as defined by Lefranc, M.-P. et al. (2003).

As previously stated, the antibody or fragment thereof may be an sdAb. The sdAb may be of camelid origin or derived from a camelid V_HH , and thus may be based on camelid framework regions; alternatively, the CDR described above may be grafted onto V_{NAR} , V_HH , V_H or V_L framework regions. In yet another alternative, the hypervariable loops described above may

5 be grafted onto the framework regions of other types of antibody fragments (Fv, scFv, Fab). The present embodiment further encompasses an antibody fragment that is "humanized" using any suitable method known in the art, for example, but not limited to CDR grafting and veneering. Humanization of an antibody or antibody fragment comprises replacing an amino acid in the sequence with its human counterpart, as found in the human consensus sequence,

10 without loss of antigen-binding ability or specificity; this approach reduces immunogenicity of the antibody or fragment thereof when introduced into human subjects. In the process of CDR grafting, one or more than one of the heavy chain CDR defined herein may be fused or grafted to a human variable region (V_H , or V_L), or to other human antibody fragment framework regions (Fv, scFv, Fab). In such a case, the conformation of said one or more than one hypervariable

15 loop is preserved, and the affinity and specificity of the sdAb for its target (i.e., toxins A and B) is also preserved. CDR grafting is known in the art and is described in at least the following: US Patent No. 6180370, US Patent No. 5693761, US Patent No. 6054297, US Patent No. 5859205, and European Patent No. 626390. Veneering, also referred to in the art as "variable region resurfacing", involves humanizing solvent-exposed positions of the antibody or

20 fragment; thus, buried non-humanized residues, which may be important for CDR conformation, are preserved while the potential for immunological reaction against solvent-exposed regions is minimized. Veneering is known in the art and is described in at least the following: US Patent No. 5869619, US Patent No. 5766886, US Patent No. 5821123, and European Patent No. 519596. Persons of skill in the art would also be amply familiar with

25 methods of preparing such humanized antibody fragments and humanizing amino acid positions.

In a specific, non-limiting example, the antibody or fragment thereof that is specific for TcdA may comprise a sequence selected from the group consisting of:

QVKLEESGGGLVQAGGSLRLSCAASGRTFNTLSMGWFRQAPGKEREFVAAVSRSGGSTYY
 30 ADSVKGRFTISRDNKNTVYLQMNSLKPEDTAVYYCAAAATKSNTTAYRLSFDYWGGGTQVT
 VSS (SEQ ID NO:34), referred to herein as A4.2;

QVKLEESGGGLVQAGGSLRLSCAASGRTFSMYRMGWFRQAPGKEREFVGVITRNGSSTYYA
 DSVKGRFTISRDNKNTVYLQMNSLKPEDTALYYCAATSGSSYLDAHVYDYWGQGTQVTVS
 S (SEQ ID NO:35), referred to herein as A5.1;

QVKLEESGGGLVQPGGSLRLSCAASGRTLSSYIVAWFRQAPGKEREFVAGISRRGGNSAYV
 ESVKGRFTISRDNANTVYLQMNSLKPEDTAVYYCAADGSVAGWGRRSVSVSSYDYWGQG
 TQVTVSS (SEQ ID NO:36), referred to herein as A19.2;

QVQLVESGGGLAQAGGSLRLSCAASGRTFSMDPMAWFRQPPGKEREFVAAGSSTGRITYY
 5 ADSVKGRFTISRDNANTVYLQMNSLKPEDTAVYYCAAAPYGANWYRDEYAYWGQGTQVT
 SS (SEQ ID NO:37), referred to herein as A20.1;

QVQLVESGGGLVQAGGSLRLSCAASIRSFNRNMGWFRQPPGKEREFVAGISWGGGSTRY
 ADSVKGRFTISRDNANTVYLQMNSLKPEDTAVYYCAAEEFGHNIATSSDEYDYWGQGTQVT
 SS (SEQ ID NO:38), referred to herein as A24.1; and

10 QVKLEESGGGLVQAGGSLRLSCAASERTFSRYPAWFRQAPGAEREFVAVISSTGTSTYYAD
 SVKGRFTISRDNANTVYLQMNNLKREDTAVYFCVNSQRTRLQDPNEYDYWGQGTQVT
 S (SEQ ID NO:39), referred to herein as A 26.8,

or a sequence substantially identical thereto.

In a specific, non-limiting example, the antibody or fragment thereof that is specific for TcdB
 15 may comprise a sequence selected from the group consisting of:

QVQLVESGGGLVQPGGSLRLSCAASGNIFSINTMGWYRQAPGKQLELVAAITSGGTTSYTD
 VEGRTISRDNANTVYLQMNSLKAEDTAVYYCNTVKVVGGRDNPDYWGQGTQVTSS
 (SEQ ID NO:40), referred to herein as B5.2;

QVKLEESGGGLVQPGGSLRLSCAASGRTASGYGMGWFRQAPGKEREFVAAISRSGAGTLN
 20 ADFVKGRFTISRDNANTVYLQMNSLKPEDTAVYYCVARPTKVD RDYATRREMYNYWGQGT
 QVTSS (SEQ ID NO:41), referred to herein as B7.3;

QVKLEESGGGLVQAGGSLRLSCSASGRTFSSGVMGWFRQAPGKQRELVAITGGSTSYTD
 SVKGRFTISRDNANTVYLQMNSLKPEDTAVYYCNSVAVVGVIKSPDYWGQGTQVTSS
 (SEQ ID NO:42), referred to herein as B13.6;

25 QVQLVESGGGSVQAGGSLRLSCAASGLSRYAMAWFRQGTGKEREFVASTNWSSGNTPYAD
 SVKGRFTISRDNANTVYLQMNSLKPEDTAIYYCAARKLDVPSRYSQHYDYWGQGTQVTSS
 (SEQ ID NO:43), referred to herein as B15.3; and

QVQLVESGGDLVQAGGSLRLSCAASGSISRISTMGWYRQAPGKQRELVAITGGTTNYAES
 VKGRFTISRDNANTMYLQMNSLKPEDTAVYYCAAGWKVVRGSLEYEYSGQGTQVTSS
 30 (SEQ ID NO:44), referred to herein as B15.5,

or a sequence substantially identical thereto

A substantially identical sequence may comprise one or more conservative amino acid mutations. It is known in the art that one or more conservative amino acid mutations to a reference sequence may yield a mutant peptide with no substantial change in physiological, chemical, physico-chemical or functional properties compared to the reference sequence; in such a case, the reference and mutant sequences would be considered "substantially identical" polypeptides. Conservative amino acid mutation may include addition, deletion, or substitution of an amino acid; a conservative amino acid substitution is defined herein as the substitution of an amino acid residue for another amino acid residue with similar chemical properties (e.g. size, charge, or polarity).

In a non-limiting example, a conservative mutation may be an amino acid substitution. Such a conservative amino acid substitution may substitute a basic, neutral, hydrophobic, or acidic amino acid for another of the same group. By the term "basic amino acid" it is meant hydrophilic amino acids having a side chain pK value of greater than 7, which are typically positively charged at physiological pH. Basic amino acids include histidine (His or H), arginine (Arg or R), and lysine (Lys or K). By the term "neutral amino acid" (also "polar amino acid"), it is meant hydrophilic amino acids having a side chain that is uncharged at physiological pH, but which has at least one bond in which the pair of electrons shared in common by two atoms is held more closely by one of the atoms. Polar amino acids include serine (Ser or S), threonine (Thr or T), cysteine (Cys or C), tyrosine (Tyr or Y), asparagine (Asn or N), and glutamine (Gln or Q). The term "hydrophobic amino acid" (also "non-polar amino acid") is meant to include amino acids exhibiting a hydrophobicity of greater than zero according to the normalized consensus hydrophobicity scale of Eisenberg (1984). Hydrophobic amino acids include proline (Pro or P), isoleucine (Ile or I), phenylalanine (Phe or F), valine (Val or V), leucine (Leu or L), tryptophan (Trp or W), methionine (Met or M), alanine (Ala or A), and glycine (Gly or G). "Acidic amino acid" refers to hydrophilic amino acids having a side chain pK value of less than 7, which are typically negatively charged at physiological pH. Acidic amino acids include glutamate (Glu or E), and aspartate (Asp or D).

Sequence identity is used to evaluate the similarity of two sequences; it is determined by calculating the percent of residues that are the same when the two sequences are aligned for maximum correspondence between residue positions. Any known method may be used to calculate sequence identity; for example, computer software is available to calculate sequence identity. Without wishing to be limiting, sequence identity can be calculated by software such as NCBI BLAST2 service maintained by the Swiss Institute of Bioinformatics, BLAST-P, Blast-N, or FASTA-N, or any other appropriate software that is known in the art.

The substantially identical sequences of the present invention may be at least 65% identical; in another example, the substantially identical sequences may be at least 65, 70, 85, 90, 95, 96, 97, 98, 99, or 100% identical, or any percentage therebetween, at the amino acid level to sequences described herein. Importantly, the substantially identical sequences retain the activity and specificity of the reference sequence. In a non-limiting embodiment, the difference in sequence identity may be due to conservative amino acid mutation(s). By way of example only, and without wishing to be limiting in any manner, the V_HHs of the present invention have between about 66% and 82% sequence identity (see Tables 5 and 6). In another non-limiting example, the present invention may be directed to an antibody or fragment thereof comprising a sequence at least 98% identical to that of the V_HHs described herein.

A substantially identical sequence as defined by the present invention also includes a mutation to introduce an additional non-canonical disulfide bond. For example, and without wishing to be limiting, the non-canonical disulfide bond may be introduced between framework region (FR) 2 and FR3. In a specific, non-limiting example, the mutation may be Ala/Gly⁵⁴→Cys⁵⁴ and/or Val/Ile⁷⁸→Cys⁷⁸ mutation (based on IMGT numbering). In a further specific example, the antibody or fragment thereof that is specific for TcdA may comprise a sequence selected from the group consisting of:

QVKLEESGGGLVQAGGSLRLSCAASGRTFNTLSMGWFRQAPGKEREFVAVSRSGGSTYY
ADSVKGRFTSRDNAKNTVYLQMNSLKPEDTAVYYCAAAATKSNTTAYRLSFDYWGGGTQV
TVSS (SEQ ID NO:45), referred to herein as A4.2m;

QVKLEESGGGLVQAGGSLRLSCAASGRTFSMYRMGWFRQAPGKEREFVIVITRNGSSTYYA
DSVKGRFTSRDNAKNTVYLQMNSLKPEDTALYYCAATSGSSYLDAHVYDYWGQGTQVTV
SS (SEQ ID NO:46), referred to herein as A5.1m;

QVKLEESGGGLVQPGGSLRLSCAASGRTLSSYIVAWFRQAPGKEREFVGISRRGGNSAYV
ESVKGRFTSRDNAKNTVYLQMNSLKPEDTAVYYCAADGSVAGWGRRSVSVSSYDYWGQG
TQVTVSS (SEQ ID NO:47), referred to herein as A19.2m;

QVQLVESGGGLAQAGGSLRLSCAASGRTFSMDPMAWFRQPPGKEREFVAGSSTGRTTY
ADSVKGRFTSRDNAKNTVYLQMNSLKPEDTAVYYCAAPYGANWYRDEYAYWGQGTQVT
VSS (SEQ ID NO:48), referred to herein as A20.1m;

QVQLVESGGGLVQAGGSLRLSCAASIRSFSNRNMGWFRQPPGKEREFV_QGISWGGGSTRY
 ADSVKGRFT_QSRDNAKKT_QVY_QLQMNSLKPEDTAVYYCAA_QEF_QGHNIATSSDEYDYWGQGTQVT
 VSS (SEQ ID NO:49), referred to herein as A24.1m;

QVKLEESGGGLVQAGGSLRLSCAASERTFSRYPVAWFRQAPGAEREFV_QVISSTGTSTYYAD
 5 SVKGRFT_QSRDNAKVT_QVY_QLQMNNLKREDTAVYFC_QAVNSQ_QRTRLQDPNEYDYWGQGTQVT
 SS (SEQ ID NO:50), referred to herein as A26.8m;

QVQLVESGGGLVQPGGSLRLSCAASGNIFSINTMGWYRQAPGKQLELV_QCAITSGGTTSYTDS
 VEGRFT_QSRDNAKNAVY_QLQMNSLKAEDTAVYYC_QNTVKVVGGRLDNPDYWGQGTQVTVSS
 (SEQ ID NO:51), referred to herein as B5.2m;

10 QVKLEESGGGLVQPGGSLRLSCAASGRTASGYGMGWFRQAPGKEREFV_QCAISRSGAGTLN
 ADFVKGRFT_QSRDNAKNT_QVY_QLQMNSLKPEDTAVYYC_QVARPTKVDRDYATRREMYNYWGQG
 TQVTVSS (SEQ ID NO:52), referred to herein as B7.3m;

QVKLEESGGGLVQAGGSLRLSCSASGRTFSSGVMGWFRQAPGKQRELV_QCAITGGSTSYTD
 SVKGRFT_QSRDNAKNT_QVY_QLQMNSLKPEDTAVYYC_QNSVAVVGGVIKSPDYWGQGTQVTVSS
 15 (SEQ ID NO:53), referred to herein as B13.6m;

QVQLVESGGGSVQAGGSLRLSCAASGLSRYAMAWFRQGTGKEREFV_QSTNWSSGNTPYAD
 SVKGRFT_QSRDNAKNT_QVY_QLQMNSLKP_QGD_QTAIYYCAA_QRKLDVPSRY_QSQHYDYWGQGTQVTVS
 S (SEQ ID NO:54), referred to herein as B15.3m; and

QVQLVESGGDLVQAGGSLRLSCAASGSISRISTMGWYRQAPGKQREL_QCATISTGGTTNYAES
 20 VKGRFT_QSRDNAKNT_QMY_QLQMNSLKPEDTAVYYCAA_QGWKVVRGSLEYEYSGQGTQVTVS
 (SEQ ID NO:55), referred to herein as B15.5m,

or a sequence substantially identical thereto, with the proviso that the substantially identical sequence retains the non-canonical disulfide bond.

The isolated or purified antibody or fragment thereof of the present invention may bind to a
 25 conformational or linear epitope. A conformational epitope is formed by amino acid residues
 that are discontinuous in sequence, but proximal in the three-dimensional structure of the
 antigen. In contrast, a linear epitope (also referred to in the art as a "sequential epitope") is
 recognized by its linear amino acid sequence, or primary structure. The conformational and
 linear epitopes of the antibodies or fragments thereof of the present invention recognize
 30 conformational and linear epitopes located in the region of TcdA responsible for cell-receptor
 binding.

The antibody or fragment thereof of the present invention may also comprise additional sequences to aid in expression, detection or purification of a recombinant antibody or fragment thereof. Any such sequences or tags known to those of skill in the art may be used. For example, and without wishing to be limiting, the antibody or fragment thereof may comprise a
5 targeting or signal sequence (for example, but not limited to ompA), a detection/purification tag (for example, but not limited to c-Myc or a His₅ or His₆), or a combination thereof. In another example, the additional sequence may be a biotin recognition site such as that described by Cronan et al in WO 95/04069 or Voges et al in WO/2004/076670. As is also known to those of skill in the art, linker sequences may be used in conjunction with the additional sequences or
10 tags, or may serve as a detection/purification tag.

The antibody or fragment thereof of the present invention may also be in a multivalent display format, also referred to herein as multivalent presentation. Multimerization may be achieved by any suitable method of know in the art. For example, and without wishing to be limiting in any manner, multimerization may be achieved using self-assembly molecules as described in
15 Zhang et al (2004a; 2004b) and WO2003/046560. The described method produces pentabodies by expressing a fusion protein comprising the antibody or fragment thereof of the present invention and the pentamerization domain of the B-subunit of an AB₅ toxin family (Merritt & Hol, 1995); the pentamerization domain assembles into a pentamer, through which a multivalent display of the antibody or fragment thereof is formed. Each subunit of the
20 pentamer may be the same or different, and may have the same or different specificity. Additionally, the pentamerization domain may be linked to the antibody or antibody fragment using a linker; such a linker should be of sufficient length and appropriate composition to provide flexible attachment of the two molecules, but should not hamper the antigen-binding properties of the antibody.

Other forms of multivalent display are also encompassed by the present invention. For example, and without wishing to be limiting, the antibody or fragment thereof may be presented as a dimer, a trimer, or any other suitable oligomer. This may be achieved by methods known in the art, for example direct linking connection (Nielson et al, 2000), c-jun/Fos interaction (de Kruif & Logtenberg, 1996), "Knob into holes" interaction (Ridgway et al, 1996).
30 Another method known in the art for multimerization is to dimerize the antibody or fragment thereof using an Fc domain, e.g., human Fc domains. The Fc domains may be selected from various classes including, but not limited to, IgG, IgM, or various subclasses including, but not limited to IgG1, IgG2, etc. In this approach, the Fc gene is inserted into a vector along with the sdAb gene to generate a sdAb-Fc fusion protein (Bell et al, 2010; Iqbal et al, 2010); the fusion
35 protein is recombinantly expressed then purified. For example, and without wishing to be

limiting in any manner, multivalent display formats may encompass chimeric formats of anti-TcdA V_HHs linked to an Fc domain, or bi- or tri-specific antibody fusions with two or three anti-TcdA V_HHs recognizing unique epitopes. Enhanced toxin neutralizing efficacy may also be obtained using various techniques, including PEGylation, fusion to serum albumin, or fusion to
5 serum albumin-specific antibody fragments; these approaches increase their blood circulation half lives, size and avidity.

The present invention also encompasses nucleic acid sequences encoding the molecules as described herein. The nucleic acid sequence may be codon-optimized for expression in various micro-organisms. The present invention also encompasses vectors comprising the
10 nucleic acids as just described. Furthermore, the invention encompasses cells comprising the nucleic acid and/or vector as described.

The present invention further encompasses the isolated or purified antibody or fragments thereof immobilized onto a surface using various methodologies; for example, and without wishing to be limiting, the antibody or fragment may be linked or coupled to the surface via His-
15 tag coupling, biotin binding, covalent binding, adsorption, and the like. Immobilization of the antibody or fragment thereof of the present invention may be useful in various applications for capturing, purifying or isolating proteins. The solid surface may be any suitable surface, for example, but not limited to the well surface of a microtiter plate, channels of surface plasmon resonance (SPR) sensorchips, membranes, beads (such as magnetic-based or sepharoseTM-
20 based beads or other chromatography resin), glass, plastic, stainless steel, a film, or any other useful surface such as nanoparticles, nanowires and cantilever surfaces.

Thus, the present invention also provides a method of capturing *Clostridium difficile* toxins, comprising contacting a sample (such as, but not limited to *C. difficile* culture supernatant, human/animal intestinal/colonic fluid, or any other suitable sample) with one or more than one
25 isolated or purified antibody or fragment thereof of the present invention. The isolated or purified antibody or fragments thereof may be immobilized onto a surface. The toxin(s) then bind to the isolated or purified antibody or fragment thereof and are thus captured. The toxins may then optionally be identified by mass spectrometric methods and/or released or eluted from their interaction with the antibody or fragment thereof; methods for releasing or eluting
30 bound molecules are well-known to those of skill in the art (for example but not limited to heat elution steps), as are spectrometric methods capable of detecting and identifying the toxin. The isolated or purified antibody or fragment thereof of the present invention provide particularly robust affinity purification reagents due to their resistance to acidic and heat elution steps.

The invention also encompasses the antibody or fragment thereof as described above linked to a cargo molecule. The cargo molecule may be any suitable molecule. For example, and without wishing to be limiting in any manner, the cargo molecule may be a detectable agent, a therapeutic agent, a drug, a peptide, an enzyme, a protease, a carbohydrate moiety, a cytotoxic agent, one or more liposomes loaded with any of the previously recited types of cargo molecules, or one or more nanoparticle, nanowire, nanotube, or quantum dots. For example, and without wishing to be limiting in any manner, the cargo molecule may be a protease that may digest the *C. difficile* toxin; in a further non-limiting example, the protease may be linked to a V_HH such as a mutant V_HH that is protease resistant. In yet another non-limiting example, the cargo molecule may be a cytotoxic agent that may be antibacterial or toxic towards host cells "infected" with *C. difficile* toxins. In a further non-limiting example, the cargo molecule is a liposome, which makes the construct well-suited as a delivery agent for mucosal vaccines. The cargo molecule may be linked to the antibody or fragment thereof by any suitable method known in the art. For example, and without wishing to be limiting, the cargo molecule may be linked to the peptide by a covalent bond or ionic interaction. The linkage may be achieved through a chemical cross-linking reaction, or through fusion using recombinant DNA methodology combined with any peptide expression system, such as bacteria, yeast or mammalian cell-based systems. Methods for linking an antibody or fragment thereof to a therapeutic agent or detectable agent would be well-known to a person of skill in the art.

The present invention also encompasses an antibody or fragment thereof linked to a detectable agent. For example, the TcdA- or TcdB-specific antibody or fragment thereof may be linked to a radioisotope, a paramagnetic label, a fluorophore, an affinity label (for example biotin, avidin, etc), fused to a detectable protein-based molecule, nucleotide, quantum dot, nanoparticle, nanowire, or nanotube or any other suitable agent that may be detected by imaging methods. In a specific, non-limiting example, the antibody or fragment thereof may be linked to a fluorescent agent such as FITC or may genetically be fused to the Enhanced Green Fluorescent Protein (EGFP). The antibody or fragment thereof may be linked to the detectable agent using any method known in the art (recombinant technology, chemical conjugation, etc.).

Thus, the present invention further provides a method of detecting *Clostridium difficile* toxins, comprising contacting a sample (such as, but not limited to *C. difficile* culture supernatant, human/animal intestinal/colonic fluid, or any other suitable sample) with one or more than one isolated or purified antibody or fragment thereof of the present invention. The isolated or purified antibody or fragments thereof may be linked to a detectable agent. The toxin(s) can

then be detected using detection and/or imaging technologies known in the art, such as, but not limited to mass spectrometric or immunoassay methods.

For example, and without wishing to be limiting in any manner, the isolated or purified antibody or fragment thereof linked to a detectable agent may be used in immunoassays (IA) including, but not limited to enzyme IA (EIA), ELISA, "rapid antigen capture", "rapid chromatographic IA", and "rapid EIA". (For example, see Planche et al, 2008; Sloan et al, 2008; Rüssmann et al, 2007; Musher et al, 2007; Turgeon et al, 2003; Fenner et al., 2008)

The present invention also encompasses the isolated or purified antibody or fragment thereof for detection of toxins in neutralized cell toxicity assays; methods for cell toxicity assays, also referred to herein as cytotoxicity assays, are known in the art and include, but are not limited to those described by Planche et al (2008); Musher et al (2007); Turgeon et al (2003); and Fenner et al (2008). Cell cytotoxicity assays involve incubating samples (for example, but not limited to patient stool samples) with cultured cells (for example, but not limited to fibroblasts) alone, or with the addition of a neutralizing agent, in this case, the isolated or purified antibody or fragment thereof as described herein. If the presence of the neutralizing agent reduces or eliminates cell toxicity observed with the cultured cells alone, presence of the toxins in the sample is confirmed. This type of assay is the practical gold standard for CDAD detection in hospital diagnostic laboratories.

The present invention also encompasses a composition comprising one or more than one isolated or purified antibody or fragment thereof as described herein. The composition may comprise a single antibody or fragment as described above, or may be a mixture of antibodies or fragments. Furthermore, in a composition comprising a mixture of antibodies or fragments of the present invention, the antibodies may have the same specificity, or may differ in their specificities; for example, and without wishing to be limiting in any manner, the antibodies or fragments may be specific to TcdA or TcdB, or a portion of the antibodies may be specific to TcdA while the other portion is specific to TcdB.

The composition may also comprise a pharmaceutically acceptable diluent, excipient, or carrier. The diluent, excipient, or carrier may be any suitable diluent, excipient, or carrier known in the art, and must be compatible with other ingredients in the composition, with the method of delivery of the composition, and is not deleterious to the recipient of the composition. The composition may be in any suitable form; for example, the composition may be provided in suspension form, powder form (for example, but limited to lyophilised or encapsulated), capsule or tablet form. For example, and without wishing to be limiting, when the composition is provided in suspension form, the carrier may comprise water, saline, a

suitable buffer, or additives to improve solubility and/or stability; reconstitution to produce the suspension is effected in a buffer at a suitable pH to ensure the viability of the antibody or fragment thereof. Dry powders may also include additives to improve stability and/or carriers to increase bulk/volume; for example, and without wishing to be limiting, the dry powder
5 composition may comprise sucrose or trehalose. In a specific, non-limiting example, the composition may be so formulated as to deliver the antibody or fragment thereof to the gastrointestinal tract of the subject. Thus, the composition may comprise encapsulation, time-release, or other suitable technologies for delivery of the antibody or fragment thereof. It would be within the competency of a person of skill in the art to prepare suitable compositions
10 comprising the present compounds.

The present invention also comprises a method of treating a *Clostridium difficile* infection, comprising administering the isolated or purified antibody or fragment thereof of the present invention, or a composition comprising the antibody or fragment thereof, to a subject in need thereof. Any suitable method of delivery may be used. For example, and without wishing to
15 be limiting in any manner, the antibody or fragment thereof, or the composition, may be delivered systemically (orally, nasally, intravenously, etc.) or may be delivered to the gastrointestinal tract. Those of skill in the art would be familiar with such methods of delivery.

The present invention will be further illustrated in the following examples. However, it is to be
20 understood that these examples are for illustrative purposes only and should not be used to limit the scope of the present invention in any manner.

Example 1: Purification of toxins and recombinant fragments

C. difficile-associated diseases (CDAD) are caused by two high-molecular weight toxins composed of enzymatic, translocation, and cell-receptor binding domains (RBD; Figure 1A).
25 TcdA and TcdB toxins were purified from natural sources, and recombinant fragments thereof were prepared.

TcdA and TcdB were isolated from *Clostridium difficile* strain 10463 (ATCC, Manassas, VA) as described previously (Keel and Songer, 2007) and were stored in 50 mM Tris-HCl buffer pH 7.5 at 4°C. The SDS-PAGE profile of the purified *C. difficile* toxins (3 µg per lane; from strain
30 10463) used is shown in Figure 1B.

Recombinant fragments of TcdA (amino acid residues 2304-2710) and TcdB (amino acid residues 2286-2366), which are fragments of the RBD, were cloned (as a *Bam*HI-*Hind*III

fragment for *tcdA* and a *Bam*HI-*Eco*RI fragment for *tcdB*) into pTrcHisB (Invitrogen, Carlsbad, CA), transforming *E. coli* DH5αMCR. Expression was induced by IPTG, cells harvested and lysed in a French pressure cell, and proteins TcdA-RBD-f1 and TcdB-RBD-f1 purified by immobilized metal-affinity chromatography (IMAC). Recombinant RBD fragments were
5 dialyzed into phosphate-buffered saline (PBS) pH 7.3 and stored at 4°C.

Example 2: Llama immunization and serum response

To isolate V_HHs which target the RBD of toxin A (TcdA) and toxin B (TcdB), a llama was immunized with recombinant RBD fragments TcdA-RBD-f1 and TcdB-RBD-f1 (Figure 1A) obtained in Example 1.

10 One male llama (*Lama glama*) was immunized by sub-cutaneous, lower-back injection of TcdA-RBD-f1 and TcdB-RBD-f1 antigens. On Day 1, 200 µg of each antigen diluted in PBS to 1 ml was injected with 1 ml of Freund's Complete Adjuvant (Sigma, St. Louis, MO). Three more injections of 100 µg of each antigen + Freund's Incomplete Adjuvant (Sigma) were performed on Days 22, 36, and 50. A final injection of 100 µg of each antigen with no adjuvant
15 was performed on Day 77. Pre-immune blood was drawn before the first injection on Day 1 and served as a negative control. Blood (10-15 ml) was collected on Days 29, 43, 57 and 84. Pre-immune and post-immune total serum was analyzed for a specific response to TcdA-RBD-f1 and TcdB-RBD-f1 by ELISA on Day 57 (see below). Llama sera from Day 84 were fractionated as previously described (Doyle et al, 2008). The resulting fractions, A1 (HCAb), A2
20 (HCAb), G1 (HCAb) and G2 (clgG) were analyzed for specific binding to TcdA-RBD-f1 and TcdB-RBD-f1 by ELISA. Briefly, 5 µg of TcdA-RBD-f1 or TcdB-RBD-f1 diluted in PBS was coated overnight (100 µl/well, 18 h, 4°C) in 96 well Maxisorp™ plates (Nalge Nunc International, Rochester, NY). Plates were blocked with bovine serum albumin (BSA), washed with PBS-T (PBS + 0.05% (v/v) Tween™-20), and serial dilutions of pre-immune total serum,
25 post-immune total serum (Day 57) and fractionated serum (Day 84) applied. After incubation at room temperature for 1.5 h and washing with PBS-T, goat anti-llama IgG (1:1,000 in PBS) was added for 1 h at 37°C. After washing with PBS-T, pig anti-goat IgG-HRP conjugate (1:3,000 in PBS) was added for 1 h at 37°C. A final PBS-T wash precluded the addition of 100 µl/well TMB substrate (KPL, Gaithersburg, MD) for 10 min. The reaction was stopped with 100 µl/well 1 M
30 H₃PO₄ and read on a BioRad plate reader (Hercules, CA) at 450 nm.

An ELISA conducted on total serum from Day 57 clearly showed a specific immune response for TcdA-RBD-f1 and TcdB-RBD-f1 compared to pre-immune sera collected before immunization on Day 0 (Figure 1C). A second ELISA performed on fractionated sera from Day 84 indicated the heavy-chain IgG (HCAb) and conventional IgG (clgG) serum fractions

recognized TcdA-RBD-f1 and TcdB-RBD-f1. For example, the G1 HCAb fraction was shown to specifically recognize both recombinant fragments and did not bind to two unrelated proteins PEB3 or CPS (Figure 1D).

Example 3: Library construction and selection of toxin-binding V_HHs

- 5 A hyperimmunized llama V_HH library was constructed based on RNA isolated from the serum collected in Example 2.

Library construction and panning was performed essentially as previously described (Arbabi-Ghahroudi et al, 2009c, 2009b; Tanha et al, 2003). Total RNA was isolated from approximately 5 x 10⁶ lymphocytes collected on day 84 post-immunization using the QIAamp RNA Blood Mini
10 Kit (QIAGEN, Mississauga, ON, Canada). About 5 µg of total RNA was used as template for first strand cDNA synthesis with oligo dT primers using the First-Strand cDNA Synthesis Kit (GE Healthcare, Baie-d'Urfé, QC, Canada). The cDNA was amplified by an equimolar mix of three variable region-specific sense primers:

MJ1: 5'-GCCCAGCCGGCCATGGCCSMKGTGCAGCTGGTGGAKTCTGGGGGA-3' (SEQ ID
15 NO:56)

MJ2: 5'-CAGCCGGCCATGGCCCAGGTAAAGCTGGAGGAGTCTGGGGGA-3' (SEQ ID
NO:57)

MJ3: 5'-GCCCAGCCGGCCATGGCCCAGGCTCAGGTACAGCTGGTGGAGTCT-3' (SEQ ID
NO:58),

- 20 and two antisense CH₂-specific primers:

CH₂: 5'-CGCCATCAAGGTACCAAGTTGA-3' (SEQ ID NO:59)

CH₂b₃: 5'-GGTACCTGTCATCCACGGACCAGCTGA-3' (SEQ ID NO:60).

Briefly, the PCR reaction mixture was set up in a total volume of 50 µl with the following components: 1-3 µl cDNA, 5 pmol of MJ1-3 primer mixture, 5 pmol of CH₂ or CH₂b₃ primers, 5
25 µl of 10x reaction buffer, 1 µl of 10 mM dNTP, 2.5 unit of Taq DNA polymerase (Hoffmann-La Roche, Mississauga, ON, Canada). The PCR protocol consisted of an (i) initial step at 94°C for 3 min, (ii) followed by 30 cycles of 94°C for 1 min, 55°C for 30 s, 72°C for 30 s and (iii) a final extension step at 72°C for 7 min. The amplified PCR products were run in a 2% agarose gel and two major bands were observed: a band of about 850 bp, corresponding to conventional
30 IgG, and a second band of around 600 bp, corresponding to heavy chain antibodies. The

smaller bands were cut and purified using the QIAquick Gel Extraction Kit (QIAGEN) and re-amplified in a second PCR in a total volume of 50 μ l using 1 μ l of DNA template, 5 pmol of each of MJ7 primer (5'-CATGTGTAGACTCGCG GCCCAGCCGGCCATGGCC-3' SEQ ID NO:61) and MJ8 primer (5'-CATGTGTAGATTCCCTGGCCGGCCTGGCCTGAGGAGACGGTGACCTGG-3' SEQ ID NO:62), 5 μ l of 10x reaction buffer, 1 μ l of 10 mM dNTP, 2.5 unit of Taq DNA polymerase. The PCR protocol consisted of (i) an initial step at 94°C for 3 min, (ii) followed by 30 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 1 min and (iii) a final extension step at 72°C for 7 min. The amplified PCR products, ranging between 340 bp and 420 bp and corresponding to V_HH fragments of heavy chain antibodies, were purified using the QIAquick PCR Purification Kit (QIAGEN), digested with *Sfi*I restriction enzyme (New England BioLabs, Pickering, ON, Canada) and re-purified using the same kit.

Eighty micrograms of pMED1 phagemid (Arbabi-Ghahroudi et al, 2009c) was digested with *Sfi*I overnight at 50°C. To minimize self-ligation, 20 units of *Xho*I and *Pst*I restriction enzymes were added and the digestion reaction was incubated for an additional 2 h at 37°C. Sixty micrograms of digested phagemid DNA was ligated with 6 μ g of digested V_HH fragments for 3 h at room temperature using LigaFast Rapid DNA Ligation System (Promega, Madison, WI) and its protocol. The ligated materials were purified using the QIAquick PCR Purification Kit in a final volume of 100 μ l and electroporated in 5 μ l portions into commercial electrocompetent TG1 *E. coli* cells (Stratagene, La Jolla, CA) as described (Arbabi-Ghahroudi et al, 2009c). The size of the library was determined (Arbabi-Ghahroudi et al, 2009c) to be 5×10^7 . Colony-PCR and sequencing involving 20 colonies showed all tested clones had unique V_HHs (Arbabi-Ghahroudi et al, 2009c). The library was grown for 2 h at 37°C, 250 rpm in the presence of 2% (w/v) glucose. The bacterial cells were pelleted, resuspended in 2xYT/Amp/Glu (2xYT medium with 100 μ g/ml ampicillin and 2% (w/v) glucose) with 35% (v/v) glycerol and stored at -80°C in small aliquots.

Panning experiments were essentially performed as described (Arbabi et al, 1997). Five milliliters of the library (1.5×10^8 cells) was thawed on ice and grown in 2xYT/Amp/Glu for about 2 h at 37°C ($A_{600} = 0.4 - 0.5$). Cells were subsequently infected with 20x excess M13KO7 helper phage (New England Biolabs) for 1 h at 37°C. The culture was then centrifuged at 4°C and infected cell pellets were resuspended in 200 ml of 2xYT/Amp with 50 μ g/ml kanamycin and incubated at 37°C and 250 rpm. The phage particles in culture supernatant were incubated with 1/5 volume of 20% PEG 6000/2.5M NaCl on ice for 1 h and centrifuged at 10,000 rpm for 15 min. The phage pellets were resuspended in 1.5 ml of sterile PBS, titrated and used as input phage for panning. For panning, 96-well Maxisorp™ plates were coated with

20 µg of TcdA-RBD-f1 or TcdB-RBD-f1 overnight at 4°C. The wells were rinsed with PBS and blocked with PBS/1% (w/v) casein for 2 h at 37°C. Approximately 10^{12} phage was added to the blocked wells and incubated for 2 h at 37°C. After 10x washing with PBS/0.1% (v/v) Tween 20, the bound phage was eluted with 0.1 M triethylamine, neutralized and mixed with exponentially growing TG1 cells. Titration of eluted phage was performed and infected bacterial cells were superinfected with M13K07 and grown overnight at 37°C. The purified phage from the overnight culture was used as the input for the next round of panning. The panning was continued for three more rounds following the same procedure except that the amount of coated RBD-fragments was reduced to 15 µg, 10 µg and 5 µg for the second, third and fourth rounds of panning, respectively.

Individual TG1 colonies obtained after round four of panning were subjected to phage ELISA screening, essentially as described elsewhere (Doyle et al, 2008), with the exception that 5 µg/ml of toxin (TcdA and TcdB) and recombinant fragments (TcdA-RBD-f1 and TcdB-RBD-f1) were coated onto microtiter plates. All positive clones were sent for DNA sequencing. Unique clones that gave high phage ELISA signals were selected for large-scale expression and purification. Seven unique TcdA-specific and 7 unique TcdB-specific binders, all determined to be V_H Hs based on the presence of characteristic amino acids at positions 42, 49, 50 and 52 (Figure 2), were selected.

Example 4: Expression and purification of selected V_H Hs

The unique TcdA-specific and TcdB-specific binders of Example 3 were sub-cloned into expression plasmids for protein expression and purification.

Phagemid vectors containing the DNA of selected V_H H clones were purified using the Qiagen MiniPrep Kit. Of the 14 V_H Hs, 11 clones were PCR amplified from the pMED1 phagemid vector with either *Bbs*I1- V_H H (5'-TATGAAGACACCAGGCCAGGTAAAGCTGGAGGAGTCT-3' SEQ ID NO:63) or *Bbs*I2- V_H H (5'-TATGA AGACACCAGGCCAGGTGCAGCTGGTGGAGTCT-3' SEQ ID NO:64) sense primers and *Bam*HI- V_H H (5'-TTGTTCCGATCCTGAGGAGACGGTGACCTG-3' SEQ ID NO:65) antisense primer. These PCR fragments were digested with *Bbs*I and *Bam*HI restriction enzymes and ligated into the similarly digested pSJF2H expression vector (Arbabi-Ghahroudi et al, 2009b). Three of the 14 clones contained internal *Bbs*I or *Bam*HI sites and were cloned into the pMED2 expression vector via digestion with *Sfi*I. The vector pMED2 is a modified version of pSJF2H which contains *Sfi*I restriction enzyme sites in its multiple cloning site. Since V_H H sequences in pMED1 are flanked with *Sfi*I restriction sites, no PCR amplification was required for sub-cloning. Upon ligation, all plasmids were transformed into electro-competent TG1 *E. coli* and

selected on LB agar plates + 100 µg/ml ampicillin. Colonies were screened by colony PCR for inserts and the DNA sequenced.

V_HHs were expressed using the 5-day M9 minimal media method (Arbabi-Ghahroudi et al, 2009c). After induction of protein expression, cell cultures were harvested at 6,000 rpm x 30 min (4°C), the supernatant decanted, and the periplasmic contents extracted from the cell pellet. Briefly, each pellet was resuspended in 30 ml of ice-cold TES buffer (0.2 M Tris-HCl buffer pH 8.0, 20% (w/v) sucrose, 0.5 mM EDTA) and incubated on ice for 30 min. Next, 40 ml of ice-cold 1/8 TES was added, incubated an additional 30 min on ice and the slurry centrifuged at 12,000 rpm for 30 min (4°C). The resulting supernatant was dialysed overnight into IMAC buffer A (10 mM HEPES buffer pH 7.0, 500 mM NaCl) and purified as previously described (Hussack et al, 2009). Eluted fractions were analyzed by SDS-PAGE and Western blotting before being dialysed into PBS. V_HH concentrations were determined by absorbance measurements at 280 nm using theoretical MW and extinction coefficients calculated with the ExPASy ProtParam Tool (Pace et al, 1995).

15 The expression of the anti-TcdA and anti-TcdB V_HH was targeted to the periplasm of TG1 *E. coli* and purified (Figure 1E) with yields ranging from 1.2 – 72.3 mg/l bacterial culture (Table 1, Example 7).

Example 5: Pentabody expression and purification

Two TcdA-specific pentameric V_HHs (pentabodies) were constructed as previously described (Zhang et al, 2004b), using the vector pVT2 which contains the verotoxin B (VTB) subunit pentamerization domain. Pentabodies were expressed in TG1 *E. coli*, as described in Example 4, the cells were lysed and processed as previously described (Hussack et al, 2009), and proteins purified with HiTrap™ IMAC columns using an imidazole gradient (0 – 500 mM) for elution. The pentabodies were constructed base on the highest affinity anti-TcdA V_HHs, A5.1 and A20.1 (Figure 3). The resulting pentabodies are referred to hereafter as A5.1p and A20.1p.

Example 6: Enzyme-linked immunosorbant assay (ELISA)

ELISA experiments were used to characterize the binding of V_HH monomers and pentamers of Examples 4 and 5, as well as their ability to recognize natural and recombinant antigen.

30 ELISA was used to determine if the purified anti-toxin V_HH monomers recognized native TcdA or TcdB and recombinant TcdA-RBD-f1 or TcdB-RBD-f1 fragments. Equivalent molar concentrations of proteins (BSA, TcdA, TcdB, TcdA-RBD-f1, and TcdB-RBD-f1) were coated overnight in 96 well microtiter plates at 4°C. The next day, wells were blocked with 3% (w/v)

skim milk diluted in PBS-T. After washing with PBS-T, purified V_HHS at concentrations as high as 10 µg/ml were added to wells with the various coated antigens for 1 h at 37°C. Wells were washed with PBS-T, rabbit anti-His₆ IgG conjugated with HRP (1:2,500 in PBS) added for 1 h at room temperature and the wells were washed an additional 5x with PBS-T. Rabbit anti-His₆ IgG-HRP did not recognize the N-terminal His₆ epitope tags on recombinant RBD fragments (data not shown). Binding was detected with TMB substrate (KPL), the reactions were stopped with 1 M H₃PO₄ and absorbance read at 450 nm. All conditions were performed in triplicate and the reported values are representative of two independent experiments.

ELISA demonstrated that 6 of 7 anti-TcdA V_HHS recognized native TcdA and TcdA-RBD-f1 and that none of the V_HHS cross-reacted with TcdB or TcdB-RBD-f1 (Figure 4A). Of the 7 anti-TcdB V_HHS tested, 4 recognized TcdB and TcdB-RBD-f1 and one clone (B5.2) also recognized TcdA (Figure 4B). ELISA experiments were also performed by coating 5 µg/ml of TcdA in microtiter wells and serially diluting V_HHS from 10 µg/ml to 1 ng/ml (Figures 4C-D). The remaining detection steps were performed as described above. With this ELISA, which was performed at a higher V_HH concentration, a fifth V_HH, B15.3, was shown to bind to TcdB.

A second ELISA was used to compare the activities of monomeric and pentameric V_HHS for TcdA. TcdA (5 µg/ml) was coated overnight in 96 well microtiter plates and blocked with 3% milk, before addition of equivalent molar concentrations of V_HH monomers and pentamers. V_HH binding to TcdA was detected with HRP-labelled rabbit-anti-His₆ IgG and TMB substrate. Binding of the pentamers to immobilized TcdA by ELISA was similar to monomeric versions of the same V_HHS (Figure 3).

Example 7: Size exclusion chromatography and surface plasmon resonance analysis

The affinity of the TcdA- and TcdB-specific V_HHS of Example 4 to their respective antigens was determined by surface plasmon resonance (SPR).

Size exclusion chromatography was performed on all purified V_HHS using a SuperdexTM 75 column (GE Healthcare) as previously described (To et al, 2005) under the control of an AKTATM FPLC. Briefly, V_HHS were applied at concentrations ranging from 0.75 – 1 mg/ml (≅ 45 – 60 µM) with a flow rate of 0.5 ml/min in a mobile phase that consisted of HBS-EP running buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.005% (v/v) P20 surfactant). As expected, all were non-aggregating monomers (Figure 5).

Fractions from the size exclusion column were then used for affinity measurements. The binding kinetics for the interaction of anti-toxin V_HHS and TcdA or TcdB were determined by surface plasmon resonance using a Biacore 3000 biosensor system (GE Healthcare). A total

of 10,377 resonance units (RUs) of TcdA and 5,980 RUs of mouse IgG 13D9 control (Liu et al, 2000) were immobilized on a CM5 sensor chip (GE Healthcare). Anti-TcdA V_HH affinity measurements were carried out in HBS-EP running buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA and 0.005% (v/v) P20 surfactant) at a flow rate of 40 µl/min. Surfaces were
5 regenerated by washing with either running buffer or 10 mM glycine pH 2.0. Initial attempts to immobilize TcdB directly onto a CM5 sensor chip were unsuccessful due to the toxin's low pI (theoretical pI = 4.42). TcdB was therefore biotinylated with the EZ-Link Sulfo-NHS-LC-LC-Biotin kit from Pierce (Rockford, IL) and 825 RUs were immobilized onto a streptavidin-coated CM5 sensor chip. However, due to the size difference of TcdB-biotin (269 kDa) compared to
10 streptavidin (53 kDa), not all streptavidin sites were occupied and roughly 1 TcdB-biotin was immobilized for every 7 streptavidin molecules. Furthermore, no binding was observed between the anti-TcdB V_HHs and immobilized TcdB-biotin. Data on the V_HH-TcdB-RBD-f1 interaction was collected by immobilizing TcdB-specific V_HHs onto the CM5 sensor chip (RUs ranging from 215 to 1209) and injecting TcdB-RBD-f1 at 20 µl/min. The IgG 13D9 or human
15 single-domain antibody HVHP420 (To et al, 2005) served as controls. In all cases, data were analyzed with BIAevaluation 4.1 software (GE Healthcare).

Surface plasmon resonance (SPR) analysis revealed 6 of 7 anti-TcdA V_HHs specifically bound TcdA with equilibrium dissociation constants ranging from 290 nM for A19.2 to 2 nM for A20.1 (Figure 6). Observed V_HH affinities for TcdA were strong, with four of the clones having *K_D*
20 values ranging from 2 to 24 nM (Table 1). The anti-TcdA V_HHs isolated here are among the highest affinity proteinaceous toxin-binding single-domain antibodies characterized to date (Stewart et al, 2007; Goldman et al, 2006; Liu et al, 2007a; Hmila et al, 2008; Goldman et al, 2008; Anderson et al, 2008).

Analyzing the TcdB-binding V_HHs by SPR was more challenging. Initial attempts to immobilize
25 TcdB onto the CM5 dextran biosensor chip may have been hindered by the low theoretical pI of TcdB. An attempt to biotinylate TcdB for immobilization on a streptavidin-coated biosensor chip was equally as unsuccessful. To circumvent this problem, anti-TcdB V_HHs were immobilized directly onto the CM5 dextran chips and data collected using various concentrations of TcdB-RBD-f1. Analyzable data could only be collected for 3 of 7 anti-TcdB
30 V_HHs, with affinity constants ranging from 100 nM to 400 nM (Figure 6; Table 1). Specific binding was detected for the other 4 anti-TcdB V_HHs, however, the data was non-analyzable (Figure 7).

Table 1. Properties of anti-*C. difficile* toxin A and B V_HH single-domain antibodies. ^aTheoretical pI calculated using the ExPASy ProtParam tool. ^b

Not neutralizing at concentration as high as 1 μM . NB: no binding detected by Biacore. NA: not attempted.

$V_{\text{H}}\text{H}$	MW (kDa)	pI ^a	Expression Yield (mg/l)	k_{on} ($\text{M}^{-1}\text{s}^{-1}$)	k_{off} (s^{-1})	K_{D} (nM)	Neutralizing?
A1.3	16.75	6.71	1.4	NB	NB	NB	No ^b
A4.2	15.73	8.59	31.3	6.7×10^5	1.6×10^{-2}	24	Yes
A5.1	15.80	6.71	55.5	1.6×10^6	5.0×10^{-3}	3	Yes
A19.2	16.01	8.61	3.8	1.4×10^4	3.9×10^{-3}	290	Yes
A20.1	16.61	6.64	72.3	8.2×10^5	1.6×10^{-3}	2	Yes
A24.1	16.71	6.71	8.5	6.0×10^4	1.6×10^{-2}	260	Yes
A26.8	16.02	6.65	64.9	1.4×10^6	1.6×10^{-2}	12	Yes
B5.2	15.11	6.04	6.7	2.0×10^3	2.0×10^{-4}	100	No ^b
B7.3	16.13	8.93	1.5	NB	NB	NB	NA
B13.2	15.79	7.98	4.0	NB	NB	NB	NA
B13.3	15.62	8.00	1.6	NB	NB	NB	NA
B13.6	15.01	8.00	3.6	2.5×10^3	1.0×10^{-3}	400	No ^b
B15.3	15.58	8.59	1.2	NB	NB	NB	NA
B15.5	15.23	7.18	4.6	2.8×10^3	1.0×10^{-3}	357	No ^b

Example 8: In vitro toxin neutralization assay

- The human lung fibroblast (HLF) cell cytotoxicity assay is routinely used for analyzing the presence of *C. difficile* toxins in biological samples (Babcock et al, 2006). The assay was used here to determine if anti-toxin $V_{\text{H}}\text{H}$ s of Example 4 or 5 could neutralize the cytopathic effects of TcdA and TcdB.

- HLF cells (ATCC#CCL-186) were purchased from ATCC (Manassas, VA) and maintained in Eagle's minimal medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen) at 37°C with 5% CO_2 . Cells were seeded in sterile 96 well microtiter plates (2×10^4 cells $200 \mu\text{l}^{-1}$ well⁻¹) for 20 h, allowing for the formation of confluent monolayers.

- Initially, a dose-response experiment was conducted to find the minimum concentration of TcdA and TcdB which induced 100% HLF cell rounding after 24 h post toxin addition. To do so, 10 μl of sterile filtered TcdA or TcdB were added to wells containing confluent monolayers, giving final toxin concentrations ranging from 500 ng/ml to 0.5 ng/ml. Each concentration was performed in triplicate and the assay performed twice. HLF cells were scored visually for rounding at various time points over 24 h. For all subsequent assays, 100 ng/ml of TcdA and 20 ng/ml of TcdB were used.

- For experiments involving $V_{\text{H}}\text{H}$ s, 20 μl of purified and sterile filtered monomeric or pentameric $V_{\text{H}}\text{H}$ s were added to HLF cells with 10 μl TcdA/B or 10 μl PBS. For experiments involving

- combinations of 2 or 3 V_HHs, the concentration of each V_HH was reduced by 1/2 and 1/3, respectively, giving the same final concentrations as experiments involving a single V_HH. Importantly, V_HHs and toxin were not pre-incubated; rather, each was added directly to HLF monolayers at time = 0 h; this was more representative of *in vivo* scenarios and did not bias
- 5 the *in vitro* results by pre-incubating. HLF cells containing PBS, V_HH, toxin, or V_HH + toxin were scored visually for cell rounding using a confocal microscope at 8 h and 24 h post antibody/toxin addition. Assays were performed in triplicate and repeated twice. Each assay was performed on fresh preparations of HLF cells (passage 3-5) and V_HHs were from separate purifications. The purified TcdA and TcdB stock remained the same for all assays.
- 10 Human lung fibroblast (HLF) cytotoxicity assays were used to determine whether V_HHs could neutralize TcdA- or TcdB-induced HLF cell rounding. Dose-response experiments with TcdA (Figure 8A) and TcdB (Figure 8B) determined the minimum toxin concentrations capable of 100% cell rounding after 24 h to be 50 ng/ml and 5 ng/ml, respectively. For all subsequent experiments, 2 x this minimum concentration was used (i.e., 100 ng/ml of TcdA and 10 ng/ml
- 15 of TcdB). V_HHs had no effect on HLF cells when incubated in the absence of toxin A (Figure 8F). When V_HHs and TcdA were added simultaneously to HLF cells, 6 of 7 anti-TcdA V_HHs inhibited TcdA-induced cell rounding in a dose-dependent manner at 8 h (data not shown) and 24 h post TcdA addition (Figure 8C-F). The neutralizing capacity of the 4 strongest monomeric V_HHs (A4.2, A5.1, A20.1 and A26.8) was similar at all concentrations tested, reflective of their
- 20 close range of K_D's (2-24 nM). The weakest neutralizers, A19.2 and A24.1, also possessed the weakest affinity constants of 290 and 260 nM, respectively. The non-binding A1.3 V_HH did not inhibit cell rounding.

The toxin neutralizing efficacy of various combinations of V_HHs was tested. All possible pair-wise and triplet combinations were tested. When various combinations of A4.2, A5.1, A20.1

25 and A26.8 were tested, their TcdA neutralizing efficacy was greater than any of the V_HHs alone. These observations suggested the V_HHs recognized distinct epitopes on TcdA, which was subsequently confirmed for A20.1 by co-injection Biacore epitope mapping experiments (Figure 10). In contrast, the other potent neutralizers appeared to bind to overlapping epitopes on TcdA. These data explain the increased neutralizing capacity seen for pairs and triplet

30 combinations containing A20.1, but do not explain why some pairs (i.e., A5.1/A26.8) or triplet combinations (i.e., A4.2/A5.1/A26.8) show greater efficacy than the individual V_HHs. The Biacore data indicated a 1:1 binding stoichiometry, which is difficult to reconcile with the observation of enhanced neutralizing efficacy with mixed V_HHs binding to overlapping epitopes. The binding stoichiometry determination assumes a mainly active toxin surface,

which may not be the case since the toxin preparations showed breakdown products (Figure 1B and Figure 9B/C).

Pentabodies A5.1p and A20.1p, were also tested for toxin neutralizing efficacy. Surprisingly, the pentabodies showed similar neutralizing efficacy to their monomeric counterparts at the
5 same concentration (data not shown). While it is possible that size effects increased the hindrance of TcdA binding to cell receptors, this effect on neutralizing potency may have been offset with a reduction in effective V_HH concentration. For example, if only 1 of 5 V_HH molecules could bind TcdA, the number of total V_HH s available for TcdA binding would be reduced by 5-fold.

10 The neutralizing capacity of the anti-TcdB V_HH s was also tested. None of the 3 TcdB-specific V_HH s were capable of TcdB neutralization, even at a concentration of 1 μ M (data not shown). It is not clear whether the V_HH affinity was insufficient for neutralization, or if the epitopes these V_HH s were raised against was not capable of preventing TcdB binding to fibroblast cell receptors. SPR Data was only collected for the TcdB-RBD-f1 – V_HH interaction; it is possible
15 that the V_HH affinities for TcdB may be considerably lower and this could account for poor neutralization.

The structure of TcdA-RBD contains 7 putative carbohydrate binding sites (Greco et al, 2006; Ho et al, 2005), which are thought to interact with epithelial cell surface receptors to mediated endocytosis (Florin and Thelestam, 1983). Due to geometric constraints, all 7 sites cannot
20 access the cell-surface receptors simultaneously, although multiple (< 7) low affinity interactions are predicted (Greco et al, 2006). As such, avidity appears to be crucial to the strength of toxin binding to its cellular receptors. It was hypothesized that pooling of neutralizing V_HH s which recognized distinct epitopes on TcdA-RBD may enhance neutralizing potency through greater hindrance of toxin-cell receptor contacts. When various combinations
25 of A4.2, A5.1, A20.1 and A26.8 were tested, their TcdA neutralizing efficacy was greater than any of the V_HH s alone. These observations suggested the V_HH s recognized distinct epitopes on TcdA, which was subsequently confirmed for one V_HH (A20.1) by co-injection Biacore epitope mapping experiments (Example 9).

Example 9: Epitope mapping

30 To gain insight into whether the TcdA-specific V_HH s recognized a linear or conformational epitope on TcdA, and whether the V_HH s could bind unique, non-overlapping epitopes, a combination of Western blotting, ELISA, and SPR was used.

Western blots using both denaturing SDS-PAGE and native PAGE, and containing TcdA were probed with anti-TcdA V_HHs or control anti-TcdA IgG (PCG4; Novus Biologicals, Littleton, CO) to determine if the V_HHs recognized linear or conformational epitopes. For denaturing SDS-PAGE Western blots, TcdA (0.75 µg/lane), A4.2 V_HH (1 µg/lane) and PCG4 IgG (1 µg/lane) were separated on 12.5% SDS-PAGE gels under reducing conditions and transferred to PVDF membranes at 100 V for 1 h. Membranes were blocked for 1 h with 5% (w/v) BSA diluted in PBS-T followed by probing with various V_HHs (25 µg/10 ml blocking buffer) or PCG4 (10 µg/10 ml blocking buffer) for 1 h. Membranes were washed 4x in PBS-T followed by addition of either: (i) mouse anti-His₆ IgG-alkaline phosphatase (AP) conjugate (Abcam, Cambridge, MA) diluted 1:5,000 in blocking buffer, (ii) HisDetector Nickel-AP conjugate (Mandel Scientific, Guelph, ON, Canada) diluted 1:5,000 in blocking buffer, or (iii) goat anti-mouse IgG-AP conjugate (Cederlane, Burlington, ON, Canada) diluted 1:10,000 in blocking buffer for 1 h. After a final set of 4 washes, membranes were subjected to AP substrate (BioRad, Hercules, CA) for 7 min, washing in dH₂O and air drying. For native PAGE Western blots, TcdA, V_HH and PCG4 (concentrations as above) were separated on 8% PAGE gels (without SDS) at 100 V for 2 h on ice. Next, gels were transferred to PVDF membranes at 20 V for 14 h at 4°C. Membranes were blocked, probed, washed and detected using the same protocol as for SDS-PAGE Western blots.

Only A19.2 V_HH recognized TcdA run under denaturing/reducing conditions (Figure 9B). The anti-TcdA mAb PCG4 (Lyerly et al, 1986), which was previously shown to recognize TcdA in Western blots (Ochsner et al, 2009), confirmed that TcdA was transferred to the blot. The weak signal obtained from A19.2 relative to PCG4 was likely due to the low affinity and/or lack of avidity of A19.2 for TcdA. In the absence of primary antibody, the secondary conjugates Nickel-AP and goat anti-mouse IgG-AP did not bind TcdA as expected. The V_HH A4.2 and PCG4 were included to confirm the functionality of the secondary conjugates. Under non-denaturing conditions (native PAGE), V_HH binding to TcdA was originally probed with anti-His₆ IgG-AP and this secondary antibody was found to cross-react with TcdA in the absence of V_HH (data not shown). To overcome this, the secondary antibody was replaced with Nickel-AP. Using this secondary conjugate, the V_HHs A4.2, A5.1, A20.1 and A26.8 recognized native TcdA while the non-binding A1.3 essentially did not react with TcdA (Figure 9C). The Nickel-AP secondary conjugate did not bind native TcdA in control blots. The diffuse signal and poor migration pattern of A4.2 V_HH control is likely due to its high pI (theoretical pI: 8.59). To confirm the presence of TcdA on native PAGE blots, PCG4 was used as a control. Just like anti-His₆ IgG-AP, the secondary antibody goat anti-mouse IgG-AP also bound TcdA in the absence of the primary probe, PCG4 in this case (Figure 9C).

To further investigate whether the V_HHs recognized linear or conformational epitopes, ELISA was performed with TcdA exposed to various temperature above and below its thermal unfolding midpoint temperature ($T_m \sim 55^\circ\text{C}$; Salnikova et al, 2008). Briefly, TcdA (5 $\mu\text{g/ml}$) was exposed to the following condition for 30 min: 4°C , 20°C , 37°C , 50°C , 60°C or 70°C . After
5 temperature treatment, 100 μl of TcdA was coated in 96 well microtiter plates overnight at 4°C and the assay performed essentially as described in Example 6, except that 0.05 – 1 $\mu\text{g/ml}$ of V_HH was used. All conditions were performed in duplicate and the reported values are representative of two independent experiments.

For 4 of the 5 V_HHs, A4.2, A5.1, A20.1 and A26.8, binding to TcdA was completely abolished
10 when TcdA was heated above its T_m (Figure 9A), confirming the above results that the V_HHs recognize a conformational epitope on TcdA. A19.2 was found to bind TcdA with the same strength at all temperature, indicating that this V_HH recognizes a linear epitope. The epitopes must be located in the region of TcdA responsible for cell-receptor binding, since llama immunization and library panning were both performed with TcdA-RBD-f1 and because the
15 V_HHs were shown to recognize this recombinant fragment by ELISA (Figure 4A).

Biacore co-injection experiments were also used to determine if the V_HHs could bind unique, non-overlapping epitopes on TcdA. Briefly, 80 μl of the first V_HH diluted in HBS-EP buffer to a concentration of 20 x its K_D was injected over 10,287 RUs of immobilized TcdA at 40 $\mu\text{l/min}$. Following injection of the first V_HH, buffer or a second V_HH (80 μl total volume, at 20 x K_D) was
20 injected at 40 $\mu\text{l/min}$ over the TcdA surface already saturated with the first V_HH. Data were collected on all possible paired combinations of A4.2, A5.1, A20.1 and A26.8, in both orientations (i.e., each V_HH acted as the first and second V_HH). Data were collected and evaluated as described in Example 7.

The observation that combining anti-TcdA V_HHs increased TcdA neutralizing efficacy relative
25 to single V_HHs at the same concentration (Figure 8C-E; Example 8) suggested the antibodies recognized distinct, non-overlapping epitopes. Co-injection Biacore experiments were performed with pairs of V_HHs, in both orientations, to determine if antibodies could bind TcdA simultaneously (Figure 10). Of the paired combinations, only those involving A20.1 V_HH showed a significant increase in response consistent with theoretical R_{max} values ($\sim 160 - 180$
30 RUs) upon co-injection. This suggests that A20.1 is free to bind TcdA when A4.2, A5.1 or A26.8 are bound at saturating concentrations and also indicates the A20.1 epitope is distinct and does not hinder binding of the other three V_HHs. For A4.2, A5.1 and A26.8, however, only minor changes in response were seen upon co-injection with theoretical R_{max} values not reached, an indication that the V_HHs were binding overlapping epitopes and hindering their

binding to TcdA (Figure 10). This was confirmed by co-injection of all three of these V_HHs simultaneously (Figure 11).

Taken together, the Biacore-based epitope mapping studies suggest A20.1 freely binds TcdA at a site that does not overlap with, or is not sterically hindered by, A4.2, A5.1 or A26.8 binding. These latter three V_HHs bind at sites on TcdA that hinder freely accessible binding of the others, suggesting these antibodies share a single epitope, or bind epitopes in such close proximity to one another that it prevents unhindered interaction with TcdA. It is likely that "overlapping" V_HHs recognize repeating epitopes. With mixed V_HHs, the spatial geometry of binding along the toxin RBD length at multiple epitope sites is such that it makes them more effective toxin neutralizers than when a single V_HH is bound. Thus, despite binding an overlapping epitope, the mixture of V_HH geometries would enhance steric effects (and neutralizing capacity) compared to single V_HH species which presents a single geometry.

Using Biacore, inhibition of the V_HH-TcdA interaction with two trisaccharides known to bind TcdA-RBD was also attempted, in order to gain insight into the TcdA epitope recognized by the V_HHs. CD-grease (α -Gal-(1,3)- β -Gal-(1,4)- β -GlcNAcO(CH₂)₈CO₂CH₃; Greco et al, 2006) and Le^X-AmHex (Gal- β 1,4-(Fuc- α 1,3)-GlcNAc-(CH₂)₆-NH₂-HOAc; P. Zhang and C.C. Ling, unpublished) are known to interact with the carbohydrate binding pockets of TcdA-RBD (Greco et al, 2006). Briefly, 4 V_HHs (i.e., A4.2, A5.1, A20.1 and A26.8) at *K_D* concentrations were injected alone or in the presence of trisaccharide (2 mM) over immobilized TcdA (~8000 RUs). The response obtained from the interaction of trisaccharide with TcdA was subtracted from response generated by V_HH + trisaccharide co-injection experiments. Then, the response of each V_HH to TcdA in the presence of trisaccharide was compared to the response generated by the V_HH-TcdA interaction. Figure 12 shows a representative example in which CD-grease and Le^X-AmHex trisaccharides are free to bind TcdA in the presence of A26.8 V_HH. We also show in Figure 13 two examples of CD-grease binding freely to TcdA in the presence of saturating amounts of bound V_HHs A20.1 (Figure 13A) and A26.8 (Figure 13B). Collectively, these studies indicate the V_HHs are not binding to TcdA in such a way as to prevent trisaccharide binding. Therefore, the anti-TcdA V_HH tested do not bind in the carbohydrate binding pocket, or sufficiently close to it, as they do not inhibit the free trisaccharide from accessing the toxin.

V_HHs have long, flexible CDR3 loop regions that have been shown to form a convex paratope that can extend into clefts or active sites of protein antigens (De Genst et al, 2006). The recently solved crystal structure of TcdA-RBD was shown to contain seven carbohydrate binding pockets thought to be involved in cell receptor binding (Greco et al, 2006). The methods just described were to investigate whether TcdA neutralization was due to V_HH

binding in the TcdA-RBD carbohydrate-binding pocket. The binding of the neutralizing V_HHs to TcdA was not inhibited in the in co-injection experiments (Figure 12). Both CD-grease and Le^X-AmHex trisaccharides were used at their predicted K_D concentrations and were shown to bind immobilized TcdA by Biacore, but did not prevent V_HH binding to TcdA when the trisaccharide response for TcdA was subtracted from response generated by co-injection experiments (Figure 12). In additional Biacore experiments, both of the trisaccharides were found to be free to bind V_HH-saturated TcdA (data not shown). Furthermore, V_HH binding to TcdA was not inhibited in ELISA with trisaccharide concentrations as high as 10 mM (data not shown). Taken together, these data suggest the present V_HHs do not inhibit free trisaccharides from accessing their binding sites on TcdA-RBD and that the V_HHs are not binding at sites occupied by the trisaccharides.

Example 10: Engineering, expression, and purification of mutant V_HHs

The extreme pH and protease-rich environment of the upper gastrointestinal tract is a major obstacle facing orally-administered protein therapeutics, including antibodies. For these reasons, the V_HHs of Example 4 were engineered to improve biophysical properties. The methods used herein are also summarized in Hussack et al (2011).

A panel of *C. difficile* toxin A (TcdA)-specific V_HHs were expressed with an additional disulfide bond by introducing Ala/Gly⁵⁴→Cys⁵⁴ and Ile⁷⁸→Cys⁷⁸ mutations. It was hypothesized that the addition of a disulfide bond in the hydrophobic core of a V_HH antibody fragments between framework 2 (FR2) and FR3 would not only increase thermal stability at neutral pH, as previously reported (Hagihara et al, 2007; Saerens et al, 2008), but would also impart resistance to proteolytic degradation and increase antibody stability at low pH. The sequences of the mutant V_HHs are shown in Figure 14.

To construct mutant V_HHs with a second internal disulfide bond, splice-overlap extension (SOE) PCR was performed essentially as described (Ho et al, 1989; Arbabi et al, 2010) using 4 primers for each V_HH and two rounds of PCR. Nucleotides encoding amino acid residues at positions 54 and 78 (IMGT numbering system) were changed to Cys-coding nucleotides through primer-forced mutation. In the first PCR, two mutagenized overlapping sub-fragments were generated for each V_HH. The primer pairs used for each V_HH were as follows: A4.2m (BbsI-VHH and A4.2mR-Cys, A4.2mF-Cys and BamHI-VHH); A5.1m (BbsI-VHH and A5.1mRCys, A4.2mFCys and BamHI-VHH); A19.2m (BbsI-VHH and A19.2mR-Cys, A19.2mF-Cys and BamHI-VHH); A20.1m (A20.1mSfil-F and A20.1mR-Cys, A20.1mF-Cys and A20.1mSfil-R); A24.1m (A20.1mSfil-F and A24.1mR-Cys, A24.1mF-Cys and A20.1mSfil-R); A26.8m (BbsI-VHH and A26.8mR-Cys, A26.8mF-Cys and BamHI-VHH). Each sub-fragment

was gel purified and spliced with its partner fragment in a second PCR. Briefly, 160 ng of each sub-fragment were added to a 50 μ l PCR mixture containing *Pfu* DNA polymerase, dNTPs and reaction buffer. The reaction was placed in a thermal cycler and the two fragments were spliced together using a program consisting of a preheating step at 94°C for 5 min and 10 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. To amplify the spliced products, the reaction was heated to 94°C for 3 min, 5 pmol (0.5 μ l) of each primer pair was added (BbsI-VHH and BamHI-VHH for A4.2m, A5.1m, A19.2m, and A26.8m; A20.1mSfiI-F and A20.1mSfiI-R for A20.1m and A24.1m), and 35 PCR cycles were performed exactly as described above. The resulting fragments were gel purified, digested with *BbsI* and *BamHI* (A4.2m, A5.1m, A19.2m, and A26.8) or *SfiI* (A20.1m and A24.1m) restriction enzymes and ligated into similarly digested expression vectors (pSJF2H or pMED2). All mutant V_HHs were expressed in the same vectors as their counterpart wild-type V_HHs (see Example 4). For cloning into pSJF2H, mutant V_HH DNA were amplified with primers containing 5' *BbsI* and 3' *BamHI* restriction sites while cloning into pMED2 required amplification with primers containing both 5' and 3' *SfiI* restriction sites. The vectors were transformed into TG1 *E. coli* for V_HH expression. Positive colonies were identified by colony-PCR and DNA sequencing, using the M13RP and M13FP primers.

Table 2: Primers used for construction of disulfide mutant V_HHs. ^aReverse and forward primers for construction of A4.2m, A5.1m, A19.2m, and A26.8m.

Primers	Sequence (5' → 3')	Purpose
M13FP	ATA AAA CGA CGG CCA GT	Screening
M13RP	CAG GAA ACA GCT ATG AC	Screening
BbsI-VHH ^a	TAT GAA GAC ACC AGG CCC AGG TAA AGC TGG AGG AGT CT	Constructing mutants
BamHI-VHH ^a	TTG TTC GGA TCC TGA GGA GAC GGT GAC CTG	Constructing mutants
A4.2mR-Cys	AGT CTG CAT AGT ATG TGC TAC CAC CAC TCC GGC TAA CAG CGC AAA CAA ACT C	Constructing A4.2m
A4.2mF-Cys	TAG CAC ATA CTA TGC AGA CTC CGT GAA GGG CCG ATT CAC CTG CTC CAG AGA C	Constructing 4.2m/A5.1m
A5.1mR-Cys	AGT CTG CAT AGT ATG TGC TAC TAC CAT TCC GGC TAA TAA CGC ATA CAA ACT C	Constructing A5.1m
A19.2mR-Cys	ACT CTA CAT AGG CAC TAT TAC CAC CAC GCC GGC TAA TAC CGC ATA CAA ACT C	Constructing A19.2m
A19.2mF-Cys	TAA TAG TGC CTA TGT AGA GTC CGT GAA GGG CCG ATT CAC CTG CTC CAG AGA C	Constructing A19.2m
A20.1mSfiI-F	ACC GTT GCG CAG GCC CAG CCG GCC ATG GCC CAG GTA CAG C	Constructing A20.1m/A24.1m
A20.1mR-Cys	TGT CTG CAT AGT ATG TGG TCC GCC CCG TAG AAC TCC CCG CGC ATA CAA ACT C	Constructing A20.1m
A20.1mF-Cys	GAC CAC ATA CTA TGC AGA CAG CGT GAA GGG CCG ATT CAC CTG CTC CAG AGA C	Constructing A20.1m
A20.1mSfiI-R	TTT CGG ATC CCT GGC CGG CCT GGC CTG AGG AGA CGG TGA CC	Constructing

		A20.1m/A24.1m
A24.1mR-Cys	AGT CTG CAT AGC GTG TGC TAC CTC CAC CCC AGC TAA TAC CGC ATA CAA ACT C	Constructing A24.1m
A24.1mF-Cys	TAG CAC ACG CTA TGC AGA CTC CGT GAA GGG CCG ATT CAC CTG CTC CAG AGA C	Constructing A24.1m
A26.8mR-Cys	AGT CTG CAT AGT ATG TGC TCG TAC CAG TCG AGC TAA TAA CGC ATA CAA ACT C	Constructing A26.8m
A26.8mF-Cys	GAG CAC ATA CTA TGC AGA CTC GGT GAA GGG CCG GTT CAC CTG CTC CAG AGA C	Constructing A26.8m

Expression and purification of mutant V_HHs was performed as described in Example 4, followed by dialysis into phosphate-buffered saline pH 7.3 (PBS), into distilled, deionized water (ddH₂O) for mass spectrometry (MS) analysis, or into 10 mM phosphate buffer pH 7.3 for

5 CD experiments. Soluble mutant V_HHs were extracted from the periplasm of TG1 *E. coli* and purified by immobilized-metal affinity chromatography (IMAC) with purified yields ranging from 3 – 12 mg/l of bacterial culture. Non-reducing SDS-PAGE and Western blot analysis of the purified products revealed the mutant V_HHs were of high purity and did not form interdomain disulfide bonds (Fig. 15A). On non-reducing SDS-PAGE gels, mutant V_HHs consistently ran

10 slower than their corresponding wild-type V_HHs (Fig. 15B).

Formation of the non-canonical disulfide linkages was confirmed by mass spectrometry analysis on cyanogen bromide + trypsin digested mutant V_HHs by identifying peptides containing the introduced disulfide bond. Briefly, 100 µl reactions containing 50 µg of mutant V_HH (diluted in PBS), 10 µl of 1 M HCl and 40 µl of CNBr (10 mg/ml stock prepared in 1 M

15 HCl) were digested for 14 h at ambient temperature in the dark. The next day, 100 µl of 1 M Tris-HCl, pH 8.6, and 60 µl of trypsin (100 µg/ml stock; sequencing grade, Roche, Mississauga, ON, Canada) were added directly to the CNBr reaction mixture and incubated for 2 h at 37°C. Samples were then analyzed by non-reducing SDS-PAGE to ensure digestion prior to MS analysis. An aliquot of the proteolytic digest of each V_HH was re-suspended in

20 0.1% formic acid (aq) and analyzed by nano-flow reversed-phase HPLC mass spectrometry (nanoRPLC-ESI-MS) using a nanoAcquity UPLC system coupled to a Q-TOF Ultima™ hybrid quadrupole/TOF mass spectrometer (Waters, Milford, MA) with data dependent analysis (DDA). The peptides were first loaded onto a 180 µm I.D. × 20 mm 5 µm symmetry®C18 trap (Waters), then eluted to a 100 µm I.D. × 10 cm 1.7 µm BEH130C18 column (Waters) using a

25 linear gradient from 0% to 36% solvent B (ACN + 0.1% formic acid) in 36 min, 36% -90% solvent B for 2 min. Solvent A was 0.1% formic acid in water. The peptide MS² spectra were searched against the V_HH protein sequences using the Mascot™ database searching algorithm (Matrix Science, London, UK). The MS² spectra of the disulphide-linked peptides were deconvoluted using the MaxEnt 3 program (Waters) for de-novo sequencing.

To precisely confirm the presence of the introduced disulfide bond, mutant V_HHs were digested with CNBr and trypsin (Fig. 16A) and their digests subjected to MS² analysis. The identification coverage of the mutant V_HHs from the analysis of their CNBr/trypsin digests using nanoRPLC-ESI-MS with DDA was more than 30%. The disulfide-linked peptide ions appeared prominent in the survey scan of the DDA experiment when the proteins were digested with a combination of CNBr and trypsin. Peptide fragments linked by the engineered Cys⁵⁴-Cys⁷⁸ disulfide bond (shown in bold text in Fig. 14) were positively identified for all mutant V_HHs by manual de-novo sequencing (Table 3). For example, the protein sequence coverage of A5.1m was 43% and a prominent ion at m/z 526.25 (3+) was sequenced as a disulfide-linked peptide EFVCVITR (P1) and FTCSR (P2) as shown (Fig. 16B, Fig. 14, Table 3). An almost complete disulfide-linked y fragment ion series was observed from one peptide with the other peptide attached as a modification via a disulfide bond, which remains intact under collision induced dissociation (CID) [59].

Table 3: Disulfide linkage determination of mutant V_HHs by MS² analysis. Mutant V_HHs were digested with CNBr and trypsin and the peptides analyzed by MS². The peptides containing the Cys⁵⁴-Cys⁷⁸ disulfide linkage are shown with connecting cysteines bolded. A nearly perfect match between MW_{for} and MW_{exp} equates to the presence of the Cys⁵⁴-Cys⁷⁸ disulfide linkage. MW_{for}: formula (expected) molecular weight (Da); MW_{exp}: experimental molecular weight (Da); ΔMW = MW_{for}-MW_{exp}.

V _H H	CNBr / tryptic peptides		MW _{for}	MW _{exp}	ΔMW
A4.2m	EFVCAVSR	FTCSR	1519.69	1519.70	-0.01
A5.1m	EFV CV ITR	FTCSR	1575.75	1575.76	-0.01
A19.2m	EFV CG ISR	FTCSR	1519.69	1519.64	0.05
A20.1m	EFV CAGS STGR	FTCSR	1722.74	1722.84	-0.10
A24.1m	EFV CGIS WGGGSTR	FTCSR	2064.91	2064.98	-0.07
A26.8m	EFV CVIS STGTSTYYADSVK	FTCSR	2766.25	2766.33	-0.08

Example 11: Size exclusion chromatography and affinity measurements of wild-type and mutant V_HHs

Wild-type and mutant V_HHs were passed over a Superdex™ 75 (GE Healthcare) size exclusion chromatography column as described in Example 7 to determine their aggregation state. Both wild-type and mutant V_HHs were determined to be non-aggregating monomers (Figures 5, 17A). Similar size exclusion profiles were obtained for mutant and wild-type, indicating the second disulfide bond does not promote the formation of interdomain disulfide-bonds or multimeric mutant V_HHs. The elution volumes (V_es) of SEC molecular weight standards are shown with arrows in Figure 17A and are aligned relative to the A4.2 and A4.2m

chromatograms. a: ovalbumin (MW = 43.0 kDa, V_e = 8.90 ml); b: carbonic anhydrase (MW = 30.0 kDa, V_e = 9.71 ml); c: trypsin inhibitor (MW = 20.1 kDa, V_e = 11.06 ml); d: α -lactalbumin (MW = 14.4 kDa, V_e = 11.97 ml); e: vitamin B (MW = 1.3 kDa, V_e = 18.7 ml). The equation of the line of a standard curve generated from these standards was $\text{LOG}_{10}\text{MW} = -0.1539V_e + 2.9949$ ($r^2 = 0.9995$). From this equation the $V_{\text{H}}\text{H}$ apparent MWs ranged from 9.8 – 13.6 kDa, indicating monomeric $V_{\text{H}}\text{H}$ s.

All kinetic rate and equilibrium constants of the mutants were determined as described (Example 7) using a Biacore 3000 instrument from GE Healthcare and 10,287 RUs of immobilized TcdA. SPR analysis revealed the specific and high-affinity binding of 4 of 6 mutant $V_{\text{H}}\text{H}$ s to TcdA (Fig. 17B, Table 4). These four were also the strongest neutralizers (see Example 14). Two mutants (A19.2m and A24.1m) exhibited non-specific binding to reference cell proteins and as a result specific interaction data could not be generated, even at antibody concentrations as high as 3.2 μM . When compared to their wild-type counterparts, the K_{D} s of 3 TcdA-binding mutants were reduced approximately 2 – 6 fold (Table 4), while the affinity of one $V_{\text{H}}\text{H}$ was relatively unchanged (K_{D} s of 24 nM and 20 nM for A4.2 and A4.2m, respectively). The K_{D} reductions were largely a result of faster k_{off} values and to a much lesser extent influenced by slower k_{on} values. Without wishing to be bound by theory, the Cys⁵⁴-Cys⁷⁸ disulfide bond may slightly distort the $V_{\text{H}}\text{H}$ structure leading to decreases in target binding affinities and decreases in antibody specificity.

Table 4: Kinetic and affinity constants of wild-type and mutant $V_{\text{H}}\text{H}$ s binding to TcdA. NB: no binding detected by Biacore, at $V_{\text{H}}\text{H}$ concentrations as high as 3.2 μM . ^aRelative to wild-type $V_{\text{H}}\text{H}$.

$V_{\text{H}}\text{H}$	Wild-type			Mutant			Fold change in K_{D} ^a
	k_{on} ($\text{M}^{-1}\text{s}^{-1}$)	k_{off} (s^{-1})	K_{D} (nM)	k_{on} ($\text{M}^{-1}\text{s}^{-1}$)	k_{off} (s^{-1})	K_{D} (nM)	
A4.2/ A4.2m	6.7×10^5	1.6×10^{-2}	24	9.3×10^5	1.9×10^{-2}	20	-1.2
A5.1/ A5.1m	1.6×10^6	5.0×10^{-3}	3	9.5×10^5	1.6×10^{-2}	17	+5.7
A19.2/ A19.2m	1.4×10^4	3.9×10^{-3}	290	-	-	-	
A20.1/ A20.1m	8.2×10^5	1.6×10^{-3}	2	6.4×10^5	5.9×10^{-3}	9.2	+4.6
A24.1/ A24.1m	6.0×10^4	1.6×10^{-2}	260	-	-	-	
A26.8/ A26.8m	1.4×10^6	1.6×10^{-2}	12	1.0×10^6	2.8×10^{-2}	28	+2.3

Example 12: Circular dichroism analysis of mutant and wild-type $V_{\text{H}}\text{H}$ s

Circular dichroism (CD) experiments were used to examine $V_{\text{H}}\text{H}$ secondary structure, tertiary structure, thermal refolding efficiency, and thermal stability at both neutral and acidic pH.

Wild-type and mutant V_{HH} s were analyzed by circular dichroism spectrophotometry using a Jasco J-815 Spectrophotometer (Jasco, Easton, MD) at pH 7.3 in 10 mM phosphate buffer (PB; 1.4 g/ml Na_2HPO_4 + 0.24 g/ml KH_2PO_4) and at pH 2.0 (10 mM PB + 50 mM HCl). For all CD experiments performed at pH 2.0, proteins were equilibrated in the above buffer for a minimum of 2 h before scanning. For far-UV CD secondary structure scans, thermal refolding, and thermal unfolding experiments, a 5 mm cuvette containing 1.5 ml of V_{HH} at 50 $\mu\text{g/ml}$ (3.2 μM ; $A_{280} \approx 0.1$) was used. In these experiments, data were collected for each sample between 190 – 260 nm with a 1 mm bandwidth, 20 nm/min scan speed and 0.5 nm data pitch. Raw data was smoothed using the Jasco software, exported and converted to mean residue ellipticity, $[\theta]$. Thermal unfolding was followed at 215 nm by far-UV CD, with CD measurements taken every 2°C from 30°C to 96°C with a temperature increase of 1°C/min. Mean residue ellipticities $[\theta]$ were used to calculate the fraction of protein folded (FF) which is shown in Equation 1,

$$\text{FF} = ([\theta] - [\theta_{\text{U}}]) / ([\theta_{\text{F}}] - [\theta_{\text{U}}]) \quad \text{Equation 1}$$

where $[\theta_{\text{F}}]$ and $[\theta_{\text{U}}]$ are the molar ellipticities of the folded (30°C) and unfolded (96°C) states, respectively. The thermal unfolding midpoint temperature (T_{m}) was obtained by plotting FF against temperature and performing sigmoidal Boltzmann curve fitting in GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). For refolding experiments, V_{HH} s were first scanned (190 nm – 260 nm) at 25°C (folded); heated at 96°C for 20 min and scanned (unfolded); and equilibrated to 25°C for 3 h before a third scan (refolded). Raw data was converted as before and thermal refolding efficiencies (TRE) were calculated at 215 nm using Equation 2,

$$\text{TRE} = (([\theta_{\text{U}}] - [\theta_{\text{R}}]) / ([\theta_{\text{U}}] - [\theta_{\text{F}}])) \times 100 \quad \text{Equation 2}$$

where $[\theta_{\text{F}}]$ is the molar ellipticity of the folded state acquired at 25°C, $[\theta_{\text{U}}]$ is the molar ellipticity of the unfolded state acquired at 96°C, and $[\theta_{\text{R}}]$ is the molar ellipticity of the refolded state acquired at 25°C. To compare the tertiary structures of wild-type and mutant V_{HH} s at neutral and acidic pH, near-UV CD experiments were performed in the range of 250 nm – 340 nm using the conditions described above with the exception that a 10 mm cuvette containing 2 ml of protein at 250 $\mu\text{g/ml}$ was used. In all cases, the ellipticity of buffer blanks were subtracted from experimental values and the reported data is the average of two independent experiments with 4 data accumulations in each.

Far-UV CD examined the V_{HH} secondary structure, and results are shown in Fig. 18. Although the overall shape of the far-UV CD spectra from wild-type and mutant V_{HH} pairs was similar at a given pH, spectra intensity shifts were observed for all wild-type/mutant pairs. In general, peak minima were seen at 216 nm – 218 nm and at 230 nm – 235 nm wavelengths but, in

almost all cases, the intensity of the peak at 216 nm – 218 nm was lower (decreased negative ellipticity) for mutant V_HHs. Another prominent feature in the far-UV CD spectra was that mutant V_HHs exhibited a near-UV shift in the peak range of 230 nm – 235 nm. Wild-type V_HHs possessed peak minima around 230 nm – 232 nm whereas mutants displayed peak minima in
 5 this region around 232 nm – 235 nm. Interestingly, A4.2/A4.2m, which had the most similar CD spectra at neutral pH of all the wild-type/mutant pairs, also had the same binding affinity for TcdA.

V_HH tertiary structures were analyzed with near-UV CD spectroscopy, and results are shown in Fig. 19. Overall, the near-UV spectra profiles were similar between wild-type and mutant V_HH
 10 pairs. Spectra from wild-type and mutant pairs shared nearly identical peak wavelengths; however, between 250 nm to 295 nm, the ellipticity of mutant V_HHs was consistently more negative than wild-type V_HHs. There were also subtle differences in peaks occurring around 297 nm, with mutant V_HHs exhibiting a minor but consistent shift to the right. Three of the four wild-type/mutant pairs (A4.2/A4.2m, A5.1/A5.1m, and A20.1m/A20.1m) produced
 15 predominantly negative ellipticity, whereas the A26.8/A26.8m pair remained positive. The contributions of the second disulfide bond cannot be ruled out as a factor which may augment the contribution of aromatic residues to ellipticity (increasing negatively) of the mutants.

Thermal refolding efficiencies (TREs) of wild-type and mutant V_HHs at neutral and acid pH were also determined by far-UV CD. CD scans were performed at 50 µg/ml concentrations of
 20 V_HHs (3.1 µM) at 25°C (folded), after heating to 96°C (unfolded), and after cooling for 3 h to 25°C (refolded). Thermal refolding efficiencies were determined as the extent to which the CD spectrum of the heated-and-cooled V_HH approached that of the folded form. At pH 7.3, the TRE of wild-type V_HHs was essentially 100% and significantly higher than for the mutants (Fig. 20, $p = 0.018$, unpaired two-tailed t -test). Specifically, wild-type V_HHs possessed a mean TRE
 25 of $99.7\% \pm 0.2\%$ compared to mutant V_HHs with a mean TRE of $90.0\% \pm 3.4\%$ (Fig. 20). The ability of V_HHs to refold in acidic conditions was also examined and, in general, mutants showed higher TREs at pH 2.0 (Fig. 20, Table 5). The mean TRE of wild-type V_HHs in acid was $68.2\% \pm 9.4\%$ compared to $80.6\% \pm 4.8\%$ for mutant TREs in acid (Fig. 20). However, the mutant TREs were not significantly higher ($p = 0.268$, unpaired two-tailed t -test). It should be
 30 noted that the TRE of 5 of 6 mutant V_HHs increased in acidic conditions, with the TRE of A26.8m reaching 64.7% compared to only 25.8% for A26.8.

Table 5: Thermal refolding efficiencies (TREs) of wild-type and mutant V_HHs at pH 2.0. TRE (%) $\pm$ SEM (n = 8).

V _H H	TRE (%)	V _H H	TRE (%)
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A4.2	80.0 ± 3.8	A4.2m	87.4 ± 1.6
A5.1	66.9 ± 4.2	A5.1m	87.2 ± 3.1
A19.2	78.3 ± 4.9	A19.2m	91.6 ± 1.4
A20.1	65.7 ± 2.5	A20.1m	85.9 ± 2.2
A24.1	92.7 ± 0.6	A24.1m	66.7 ± 0.3
A26.8	25.8 ± 17.6	A26.8m	64.7 ± 2.2

Temperature-induced unfolding experiments were conducted in order to determine $V_{\text{H}}H$ T_{m} s and T_{onset} s by following changes in $V_{\text{H}}H$ ellipticity at 215 nm (Fig. 21; Tables 6, 7). All $V_{\text{H}}H$ s exhibited sigmoidal melting curves. The wild-type $V_{\text{H}}H$ s have high T_{m} s (as high as 84.7°C) - significantly higher than those reported for other $V_{\text{H}}H$ s [60]. For all six $V_{\text{H}}H$ s, the mutants possessed higher T_{m} values, at both neutral and acidic pH (Figure 21 and Table 6). At neutral pH, the T_{m} values of mutants ranged from 78.8°C to 93.6°C, with one mutant, A5.1m, having a T_{m} 11.6°C higher than wild-type (A5.1). The increase in mutant $V_{\text{H}}H$ T_{m} s relative to wild-type ranged from 3.7°C to 11.6°C. Overall, at neutral pH, the mean $T_{\text{m}} \pm \text{SEM}$ was 76.2°C ± 1.8°C and 83.6°C ± 2.3°C for wild-type and mutant $V_{\text{H}}H$ s, respectively (Fig. 21B). At acidic pH a considerable reduction in T_{m} was observed for both wild-type (22.1°C to 32.4°C) and mutant $V_{\text{H}}H$ s (23.7°C to 31.2°C) when compared to the T_{m} values recorded at pH 7.3. However, at acidic pH the T_{m} of all six mutants was still significantly higher than the corresponding wild-type $V_{\text{H}}H$ s ($p = 0.002$, unpaired two-tailed t -test). In acid, the increase in mutant $V_{\text{H}}H$ T_{m} s relative to wild-type ranged from 2.1°C to 11.6°C, which is a nearly identical spread in temperature increases to that seen at neutral pH. Overall, at pH 2.0, the mean $T_{\text{m}} \pm \text{SEM}$ was 49.3°C ± 1.2°C and 56.6°C ± 1.2°C for wild-type and mutant $V_{\text{H}}H$ s, respectively (Fig. 21B). Interestingly, the highest T_{m} gains at both pH were seen for the four strongest neutralizers. The T_{m} differences between wild-type/mutant pairs are more significant at acidic pH than neutral pH. Without wishing to be bound by theory, these results (Table 6; Fig. 21) suggest the Cys⁵⁴-Cys⁷⁸ disulfide bond may stabilize the $V_{\text{H}}H$ s from acid-induced denaturation.

For example, A5.1 wild-type $V_{\text{H}}H$ had a T_{m} of 73.1°C and 45.6°C at neutral and acidic pH, respectively, while the A5.1 mutant (A5.1m) had a T_{m} of 84.7°C and 57.2°C at neutral and acidic pH, respectively.

Table 6: Comparison of wild-type (WT) and mutant (Mut) $V_{\text{H}}H$ thermal unfolding midpoint temperatures (T_{m}) at neutral and acidic pH. * Minimum estimated T_{m} .

$V_{\text{H}}H$	T_{m} (°C) at pH 7.3			T_{m} (°C) at pH 2.0		
	Wild-type	Mutant	ΔT_{m}	Wild-type	Mutant	ΔT_{m}
A4.2/ A4.2m	84.7*	93.6*	8.9	52.3	62.4	10.1
A5.1/ A5.1m	73.1	84.7*	11.6	45.6	57.2	11.6
A19.2/ A19.2m	75.1	78.8	3.7	53.0	55.1	2.1

A20.1/ A20.1m	72.4	79.1	6.7	46.6	55.4	8.8
A24.1/ A24.1m	74.6	80.1	5.5	49.4	54.6	5.2
A26.8/ A26.8m	77.2	85.3*	8.1	48.8	54.8	6.0

Using the thermal unfolding curves, V_HH T_{onset} temperatures were also identified; this is the temperature at which 5% of the V_HH was unfolded (Fig. 21C; Table 7). The T_{onset} of mutant V_HH s was significantly higher than wild-type V_HH s at both neutral and acidic pH ($p = 0.027$ and $p = 0.006$, respectively, unpaired two-tailed t -test). The T_{onset} differences between wild-type/mutant pairs are more significant at acidic pH than neutral pH. At pH 7.3, the mean $T_{onset} \pm SEM$ was $68.9^\circ C \pm 1.8^\circ C$ and $74.9^\circ C \pm 1.5^\circ C$ for wild-type and mutant V_HH s, respectively. At pH 2.0, the mean $T_{onset} \pm SEM$ was $41.2^\circ C \pm 1.3^\circ C$ and $47.3^\circ C \pm 1.3^\circ C$ for wild-type and mutant V_HH s, respectively. Therefore, the lowest T_{onset} for the mutants was $45.0^\circ C$, whereas two of the wild-type V_HH s (A5.1, A20.1) already had T_{onset} s of $\sim 37^\circ C$ at pH 2.0 (physiological stomach conditions).

Table 7: Onset temperatures (T_{onset} s) of wild-type and mutant V_HH s. T_{onset} is defined as the temperature at which 5% of the V_HH is unfolded.

V_HH	T_{onset} pH 7.3 ($^\circ C$)		T_{onset} pH 2.0 ($^\circ C$)	
	Wild-type	Mutant	Wild-type	Mutant
A4.2/ A4.2m	76.5	80.0	43.7	53.1
A5.1/ A5.1m	65.2	76.6	37.8	48.4
A19.2/ A19.2m	68.3	71.4	45.3	45.0
A20.1/ A20.1m	64.6	72.0	37.8	46.3
A24.1/ A24.1m	68.2	71.7	42.2	46.0
A26.8/ A26.8m	70.7	77.8	40.3	45.2

15 Example 13: Protease resistance profile analysis of mutant and wild-type V_HH s

The sensitivity of wild-type and mutant V_HH s to the three major gastrointestinal proteases pepsin, trypsin, and chymotrypsin, was explored to determine whether the Cys⁵⁴-Cys⁷⁸ disulfide bond improved V_HH resistance to proteolytic degradation. The effects of the proteases were analyzed by SDS-PAGE and MS analysis.

20 All reactions were performed in 20 μ l volumes with 4.8 μ g of V_HH diluted in PBS pH 7.3. For pepsin digestions, reactions contained 17 μ l of V_HH , 2 μ l of porcine stomach pepsin (460 U/mg; Sigma), and 1 μ l of 1 M HCl (final pH = 2.0). Final pepsin concentrations in each reaction ranged from 0.1 μ g/ml to 100 μ g/ml. Digestions were incubated at $37^\circ C$ for 60 min and neutralized with 1 μ l of 1 M NaOH. For trypsin and chymotrypsin digestions, reactions
25 contained 18 μ l of V_HH (diluted in PBS supplemented with 10 mM $CaCl_2$) and 2 μ l of either

trypsin or chymotrypsin (sequencing grade, Hoffmann-La Roche). Final trypsin/chymotrypsin concentrations ranged from 0.1 µg/ml to 100 µg/ml. Digestions were incubated at 37°C for 60 min and neutralized with 1 µl of protease inhibitor cocktail (Sigma). All neutralized V_HH-protease reactions and controls (V_HHs with no protease) were separated by SDS-PAGE, stained with Coomassie and photographed using an Alphamager3400 (Alpha Innotech Corporation, San Leandro, CA). To determine the percent of V_HH retained after protease digestions, densitometry analysis was performed using the AlphaEaseFc software package (Version 7.0.1, Alpha Innotech Corporation) on control and digested V_HHs. A total of three independent digestion reactions were performed on all of the V_HHs at each protease concentration and each were run on separate SDS-PAGE gels. Digestions at the highest protease concentration (100 µg/ml) that were not analyzed by SDS-PAGE were buffer exchanged into ddH₂O using Millipore Biomax 5K MWCO spin columns (Millipore, Billerica, MA) and subjected to MS analysis to identify the cleavage products, or analyzed by SPR for TcdA binding activity.

Protease concentrations of 0.1 µg/ml, 1 µg/ml, 10 µg/ml, and 100 µg/ml were explored. When the lowest concentrations of proteases (0.1 µg/ml and 1 µg/ml) were used in digestion reactions, wild-type and mutants appeared similar to undigested controls on SDS-PAGE (data not shown). Similarly, V_HHs were only moderately susceptible to protease degradation at 10 µg/ml (data not shown). In order to see clear differences in the proteolytic susceptibility of wild-type and mutant V_HHs, all remaining digestions were performed at protease concentrations of 100 µg/ml.

A representative SDS-PAGE gel comparing A5.1 wild-type and mutant V_HH digestion with various concentrations of pepsin is shown in Figure 22A. A reduction in V_HH size from ~16 kDa (control) to either ~14 kDa, or complete digestion to smaller fragments can be observed.

The band at ~14 kDa routinely appeared in digestions with each of the proteases and was shown by MS mass analysis to correspond to cleavage at various positions within the V_HH C-terminal c-Myc epitope tag. Loss of the epitope tag corresponded to reductions of 1641.7 Da, 1754.8 Da, and 1641.7 Da for pepsin, trypsin, and chymotrypsin digested V_HHs, respectively (data not shown). Overall, significant increases in pepsin resistance were found for all mutant V_HHs compared to their wild-type counterparts ($p = 0.026$, Mann-Whitney U test) (Fig. 22B, E; Table 8). All mutant V_HHs were found to possess greater pepsin resistance, a protease that functions at acidic pH, compared to the wild-type V_HHs ($p = 0.026$, Mann-Whitney U test) (Figure 22B, E and Table 8). The increase in mutant V_HH pepsin resistance relative to corresponding wild-type ranged from almost 4.5% to 63% (Table 8). For example, A5.1 was completely degraded after incubation with pepsin, while nearly 50% of A5.1m remained intact

(Fig. 22A, B). The biggest increase in pepsin resistance was found for A4.2m, where an almost 63% increase in intact V_HH structure was found relative to A4.2. Interestingly, A4.2m also had the highest T_m and T_{onset} at pH 2.0 (Table 7), the same pH at which the pepsin digestions were performed.

- 5 In general, V_HHs with a higher T_m at pH 2 correlated with a greater resistance to pepsin degradation (Figure 23A) ($R^2 = 0.735$). Thus, while wild type V_HHs with lower T_m s occupied the low protease resistance region of the graph, the mutants with higher T_m s occupied the high protease resistance region of the graph. There was also a moderate correlation between V_HH pepsin resistance and T_m s at pH 7.3 ($r^2 = 0.500$, data not shown). In addition, a strong
- 10 correlation between wild-type V_HH pepsin resistance and wild-type V_HH T_{onset} at pH 2.0 was noted ($r^2 = 0.975$, Fig. 23B). No correlation was evident between mutant V_HH pepsin resistance and mutant V_HH T_{onset} at pH 2.0 ($r^2 = 0.191$, data not shown); without wishing to be bound by theory, this was likely because mutant V_HH T_{onset} temperatures were much higher than the temperature at which pepsin digestions were performed (37°C).
- 15 **Table 8:** Comparison of wild-type (WT) and mutant (Mut) V_HH resistance profiles to the major gastrointestinal proteases. All V_HH digestions were performed at 37°C for 1 hour in the presence of 100 µg/ml of protease; resistance profiles were obtained by comparing the intensity of protease-digested V_HHs relative to untreated controls using SDS-PAGE and imaging software; values represent the mean ± SEM of three independent experiments.

V _H H	Pepsin Resistance (%)		Trypsin Resistance (%)		Chymotrypsin Resistance (%)	
	WT	Mut	WT	Mut	WT	Mut
A4.2	11.08 ± 1.88	73.87 ± 7.23	35.72 ± 7.08	4.80 ± 0.61	13.60 ± 6.50	3.18 ± 1.10
A5.1	0.53 ± 0.15	46.63 ± 1.99	96.23 ± 7.09	83.30 ± 4.96	14.03 ± 3.15	27.00 ± 4.05
A19.2	30.37 ± 3.16	52.27 ± 0.32	0.73 ± 0.73	0.27 ± 0.27	8.30 ± 1.14	0.18 ± 0.10
A20.1	0.68 ± 0.68	5.04 ± 0.76	72.77 ± 4.85	82.80 ± 1.97	10.17 ± 1.85	16.17 ± 5.26
A24.1	10.45 ± 2.39	36.02 ± 1.11	75.03 ± 9.63	66.50 ± 3.58	22.03 ± 5.01	43.80 ± 2.08
A26.8	3.17 ± 1.24	24.56 ± 1.45	2.03 ± 2.03	4.10 ± 1.27	8.40 ± 1.23	40.83 ± 8.81

- 20 The resistance profiles of wild-type and mutant V_HHs to trypsin and chymotrypsin, proteases that function at neutral pH, were more varied without a clearly defined trend (Figure 22C-E and Table 8). 4 of 6 mutant V_HHs showed increased resistance to chymotrypsin, with significant increases found in clones A5.1m, A24.1m, and A26.8m ($p < 0.05$) compared to their wild-type
- 25 counterparts. No statistical differences were found between trypsin digested wild-type and mutant V_HHs (Fig. 22C-E; Table 8), except for A4.2m, where trypsin resistance was actually reduced from almost 36% in the wild-type V_HH to almost 5% in the mutant. Both the wild-type

and mutant versions of A19.2 and A26.8 were very susceptible to trypsin degradation. No correlation was evident between V_HH trypsin resistance and T_m s at pH 7.3 or pH 2.0 ($r^2 = 0.138$ and $r^2 = 0.138$, respectively) or between V_HH chymotrypsin resistance and T_m s at pH 7.3 or pH 2.0 ($r^2 = 0.012$ and $r^2 = 0.004$, respectively).

- 5 The ability of pepsin-treated mutants (A4.2m, A5.1m, A20.1m, and A26.8m) to bind TcdA was evaluated by SPR. SPR analyses confirmed the pepsin-treated mutants ("V_HH – tag") retained TcdA binding as their k_{off} values (Fig. 23C) were essentially the same as those of untreated controls (Table 4; Fig. 23C). SPR analysis on pepsin-digested wild-type V_HHs could not be performed since these V_HHs were significantly degraded by pepsin. Without wishing to be
10 bound by theory, this highlights the impact a second disulfide bond in the hydrophobic core has on V_HH conformational stability at low pH and resistance to proteolytic degradation by pepsin.

Example 14: TcdA toxin neutralization assay

- In vitro* TcdA neutralization assays were performed essentially as described [20]. Human lung
15 fibroblast cell rounding was reported 24 h post addition of TcdA (100 ng/ml), TcdA + wild-type V_HH (1000 nM) or TcdA + mutant V_HH (1000 nM). Specifically, V_HHs were added as pooled mixtures of A4.2, A5.1, A20.1, and A26.8 (250 nM each, 1000 nM total) or A4.2m, A5.1m, A20.1m, and A26.8m (250 nM each, 1000 nM total). The percentage of cell rounding was scored visually using light microscopy and the reported values are the average of two
20 independent experiments in which each V_HH mixture was tested in triplicate.

- Mutant V_HHs retained their ability to neutralize to cytotoxic effects of TcdA on monolayers of fibroblast cells. Comparison of the neutralization capacity of pooled mixtures (1000 nM total) of wild-type and mutant V_HHs revealed mutants performed nearly as well as wild-types at reducing TcdA-mediated cell rounding (Fig. 24). Given that 3 of 4 mutants showed weaker
25 affinity for TcdA the reduction in neutralizing capacity relative to wild-type V_HHs was not unexpected.

- As indicated above, the mutant antibodies were compared to their wild-type counterparts with respect to expression, yield, solubility, affinity for TcdA, thermal unfolding at neutral and acidic
30 pH, and protease resistance. Mutant V_HHs were found to be soluble, non-aggregating monomers, possessing similar affinity constants to that of WT V_HHs. A significant increase in the midpoint temperature of unfolding (4 - 12°C) was observed for all mutants, at both neutral and acidic pH ($p < 0.05$; unpaired two-tailed t test). Digestion of the V_HHs with major

- gastrointestinal proteases at biologically relevant concentrations revealed a significant increase in pepsin resistance for all mutants ($p < 0.05$; unpaired two-tailed Mann-Whitney U test). However, increases in resistance profiles to chymotrypsin and trypsin were not as universal. Overall, the introduction of an additional disulfide bond in the hydrophobic core of the anti-TcdA V_H Hs not only increased thermal stability at neutral pH, but also represents a generic strategy to increase antibody stability at low pH and impart pepsin resistance which is desirable for protein-based oral therapeutics.

Example 15: Sequence identities between V_H Hs

Sequence identities between the toxin A binders, as well as toxin B binders were determined.

- 10 The sequences of V_H H pairs were aligned using ClustalW (Thompson et al, 1994), and the percentage identity between the V_H H pairs was calculated using the BioEdit Sequence Alignment Editor. Results are shown in Tables 9 and 10, below.

Table 9: Percentage amino acid sequence identities between TcdA-binding V_H Hs.

V_H H 1	V_H H 2	Identity (%)
A4.2	A5.1	82
A4.2	A19.2	76
A4.2	A20.1	82
A4.2	A24.1	78
A4.2	A26.8	75
A5.1	A19.2	77
A5.1	A20.1	78
A5.1	A24.1	77
A5.1	A26.8	78
A19.2	A20.1	77
A19.2	A24.1	75
A19.2	A26.8	75
A20.1	A24.1	80
A20.1	A26.8	77
A24.1	A26.8	74
A4.2	A4.2m	98.4
A5.1	A5.1m	98.4
A19.2	A19.2m	98.4
A20.1	A20.1m	98.4
A24.1	A24.1m	98.4
A26.8	A26.8m	98.4

- 15 **Table 10:** Percentage amino acid sequence identities between TcdB-binding V_H Hs.

V_H H 1	V_H H 2	Identity (%)
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B5.2	B7.3	69
B5.2	B13.6	81
B5.2	B15.3	66
B5.2	B15.5	73
B7.3	B13.6	74
B7.3	B15.3	72
B7.3	B15.5	69
B13.6	B15.3	68
B13.6	B15.5	71
B15.3	B15.5	69
B5.2	B5.2m	98.3
B7.3	B7.3m	98.4
B13.6	B13.6m	98.3
B15.3	B15.3m	98.4
B15.5	B15.5m	98.3

The embodiments and examples described herein are illustrative and are not meant to limit the scope of the invention as claimed. Variations of the foregoing embodiments, including alternatives, modifications and equivalents, are intended by the inventors to be encompassed
5 by the claims. Furthermore, the discussed combination of features might not be necessary for the inventive solution.

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CLAIMS:

1. An isolated or purified antibody or fragment thereof selected from the group consisting of:

an isolated or purified antibody or fragment thereof comprising a sequence of CDR 1 of GRTFNTLS (SEQ ID NO:1); CDR2 of VSRSGGST (SEQ ID NO:7); and CDR3 of AAAATKSNTTAYRLSFDY (SEQ ID NO:13);

an isolated or purified antibody or fragment thereof comprising a sequence of CDR 1 of GRTFSMYR (SEQ ID NO:2); CDR2 of ITRNGSST (SEQ ID NO:8); and CDR3 of AATSGSSYLDAAHVYDY (SEQ ID NO:14);

an isolated or purified antibody or fragment thereof comprising a sequence of CDR 1 of GRTLSSYI (SEQ ID NO:3); CDR2 of ISRRGGNS (SEQ ID NO:9); and CDR3 of AADGSVAGWGRRSVSVSSYDY (SEQ ID NO:15);

an isolated or purified antibody or fragment thereof comprising a sequence of CDR 1 of GRTFSMDP (SEQ ID NO:4); CDR2 of GSSTGRTT (SEQ ID NO:10); and CDR3 of AAAPYGANWYRDEYAY (SEQ ID NO:16);

an isolated or purified antibody or fragment thereof comprising a sequence of CDR 1 of IRSFSNRN (SEQ ID NO:5); CDR2 of ISWGGGST (SEQ ID NO:11); and CDR3 of AAIEFGHNIATSSDEYDY (SEQ ID NO:17); and

an isolated or purified antibody or fragment thereof comprising a sequence of CDR 1 of ERTFSRYP (SEQ ID NO:6); CDR2 of ISSTGTST (SEQ ID NO:12); and CDR3 of AVNSQRTRLQDPNEYDY (SEQ ID NO:18)

wherein the antibody or fragment thereof is specific to TcdA.

2. The isolated or purified antibody or fragment thereof of claim 1, respectively comprising a sequence selected from the group consisting of:

QVKLEESGGGLVQAGGSLRLSCAASGRTFNTLSMGWFRQAPGKEREFVAAVSRSGGSTY
YADSVKGRFTISRDNKNTVYLMNSLKPEDTAVYYCAAAATKSNTTAYRLSFDYWGQGTQ
VTVSS (SEQ ID NO:34);

QVKLEESGGGLVQAGGSLRLSCAASGRTFSMYRMGWFRQAPGKEREFGVITRNGSSTY
YADSVKGRFTISRDNANTVYLQMNSLKPEDTALYYCAATSGSSYLDAAHVYDYWGQGTQ
VTVSS (SEQ ID NO:35);

QVKLEESGGGLVQPGGSLRLSCAASGRTLSSYIVAWFRQAPGKEREFGVAGISRRGGNSAY
VESVKGRFTISRDNANTVYLQMNSLKPEDTAVYYCAADGSVAGWGRRSVSVSSYDYWG
QGTQVTVSS (SEQ ID NO:36);

QVQLVESGGGLAQAGGSLRLSCAASGRTFSMDPMAWFRQPPGKEREFGVAAGSSTGRTTY
YADSVKGRFTISRDNANTVYLQMNSLKPEDTAVYYCAAAPYGANWYRDEYAYWGQGTQ
VTVSS (SEQ ID NO:37);

QVQLVESGGGLVQAGGSLRLSCAASIRSFNRNMGWFRQPPGKEREFGVAGISWGGGSTR
YADSVKGRFTISRDNANTVYLQMNSLKPEDTAVYYCAAEEFGHNIATSSDEYDYWGQGTQV
TVSS (SEQ ID NO:38);

QVKLEESGGGLVQAGGSLRLSCAASERTFSRYPVAWFRQAPGAEREFGVAVISSTGTSTYYA
DSVKGRFTISRDNANTVYLQMNNLKREDTAVYFCVNSQRTRLQDPNEYDYWGQGTQVT
VSS (SEQ ID NO:39);

QVKLEESGGGLVQAGGSLRLSCAASGRTFNTLSMGWFRQAPGKEREFGCAVSRSGGSTY
YADSVKGRFTCSRDNANTVYLQMNSLKPEDTAVYYCAAATKSNTTAYRLSFDYWGQGT
QVTVSS (SEQ ID NO:45);

QVKLEESGGGLVQAGGSLRLSCAASGRTFSMYRMGWFRQAPGKEREFGCVITRNGSSTY
YADSVKGRFTCSRDNANTVYLQMNSLKPEDTALYYCAATSGSSYLDAAHVYDYWGQGTQ
VTVSS (SEQ ID NO:46);

QVKLEESGGGLVQPGGSLRLSCAASGRTLSSYIVAWFRQAPGKEREFGVAGISRRGGNSAY
VESVKGRFTCSRDNANTVYLQMNSLKPEDTAVYYCAADGSVAGWGRRSVSVSSYDYWG
QGTQVTVSS (SEQ ID NO:47);

QVQLVESGGGLAQAGGSLRLSCAASGRTFSMDPMAWFRQPPGKEREFGVAGSSTGRTTY
YADSVKGRFTCSRDNANTVYLQMNSLKPEDTAVYYCAAAPYGANWYRDEYAYWGQGTQ
VTVSS (SEQ ID NO:48);

QVQLVESGGGLVQAGGSLRLSCAASIRSFSNRNMGWFRQPPGKEREFVCGISWGGGSTR
YADSVKGRFTCSRDNAAKKTIVYLQMNSLKPEDTAVYYCAAIEFGHNIAITSSDEYDYWGQGTQ
VTVSS (SEQ ID NO:49); and

QVKLEESGGGLVQAGGSLRLSCAASERTFSRYPVAWFRQAPGAEREFVVISSTGTSTYYA
DSVKGRFTCSRDNAAKKTIVYLQMNNLKREDTAVYFCAVNSQRTRLQDPNEYDYWGQGTQV
TVSS (SEQ ID NO:50),

or a sequence having at least 65% sequence identity thereto.

3. The isolated or purified antibody or fragment thereof of any one of claims 1 and 2, wherein the antibody is a single-domain antibody (sdAb).
4. The isolated or purified antibody or fragment thereof of claim 3, wherein the sdAb is of camelid origin.
5. The isolated or purified antibody or fragment thereof of any one of claims 1 to 4, wherein the antibody or fragment thereof binds to a conformational or linear epitope.
6. The isolated or purified antibody or fragment thereof of any one of claims 1 to 5, wherein the antibody or fragment thereof is in a multivalent display format.
7. A nucleic acid molecule encoding the isolated or purified antibody or fragment thereof of any one of claims 1 to 6.
8. A vector comprising the nucleic acid molecule of claim 7.
9. The isolated or purified antibody or fragment thereof of any one of claims 1 to 6, wherein the antibody or fragment thereof is immobilized onto a surface.
10. The isolated or purified antibody or fragment thereof of any one of claims 1 to 6, wherein the antibody or fragment thereof is linked to a cargo molecule.
11. The isolated or purified antibody or fragment thereof of claim 10, wherein the cargo molecule is a detectable agent, a therapeutic, a drug, a peptide, a carbohydrate moiety, an enzyme, or a cytotoxic agent; one or more liposomes loaded with a detectable agent, a therapeutic, a drug, a peptide, an enzyme, or a cytotoxic agent; or one or more nanoparticles, nanowires, nanotubes, or quantum dots.

12. A composition comprising one or more than one isolated or purified antibody or fragment thereof of any one of claims 1-6, 10 and 11 and a pharmaceutically-acceptable carrier, diluent, or excipient.

13. A use of the isolated or purified antibody or fragment thereof of any one of claims 1-6, 10 and 11 or the composition of claim 12 to treat a *Clostridium difficile* infection in a subject in need thereof.

14. A method of capturing *Clostridium difficile* TcdA toxin, comprising contacting a sample with one or more than one isolated or purified antibody or fragment thereof of claim 9, and allowing the toxin(s) to bind to the isolated or purified antibody or fragment thereof.

15. The method of claim 14, further comprising identification of the toxin by a mass spectrometric method.

16. The method of claim 14 or 15, further comprising eluting the bound toxin.

17. The method of claim 16, wherein elution of the bound toxin is accomplished by heat elution.

18. A method of detecting *Clostridium difficile* TcdA toxin, comprising contacting a sample with one or more than one isolated or purified antibody or fragment thereof of claim 10 or 11, and detecting the bound antibody or fragment thereof using a suitable detection and/or imaging technology.

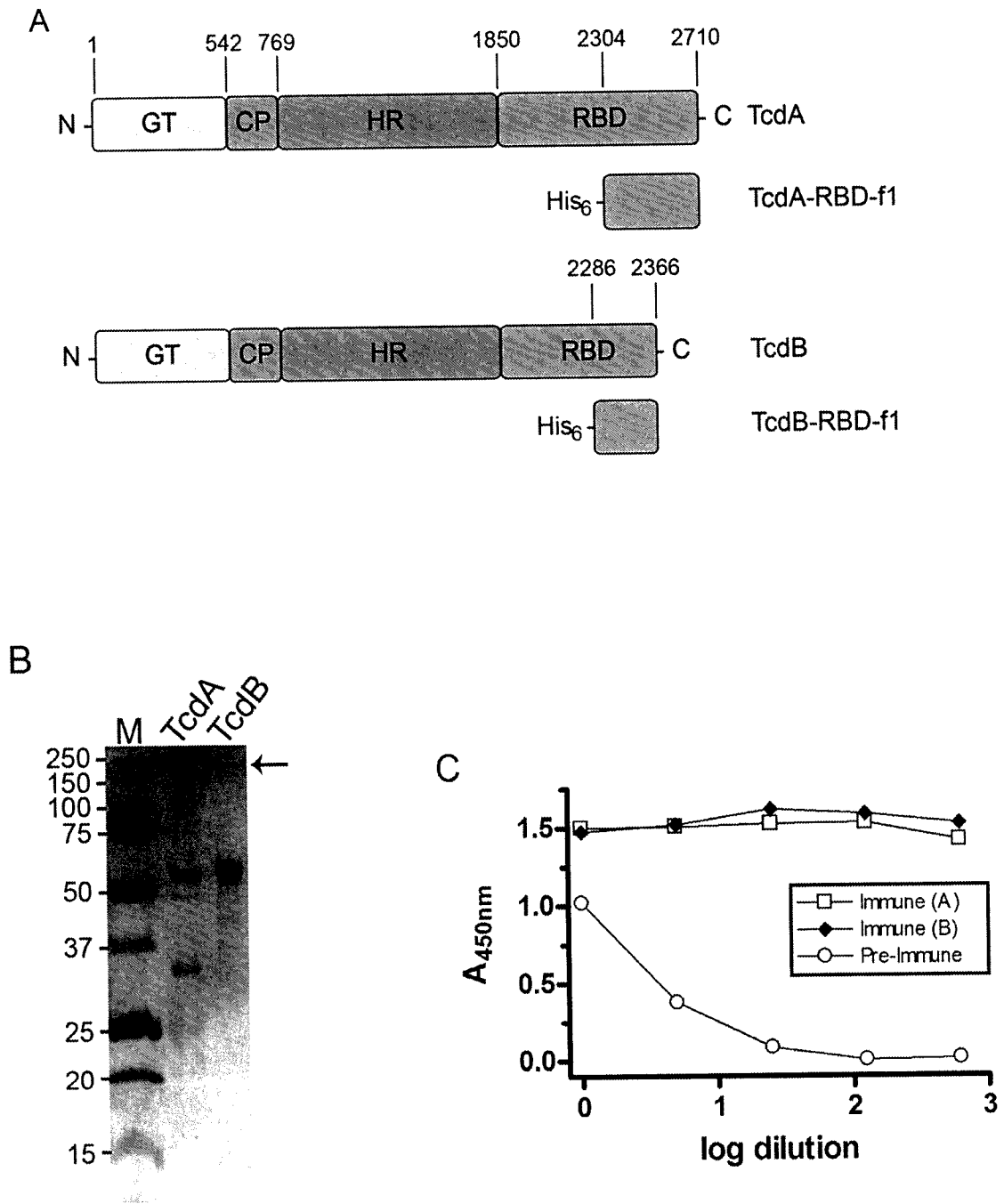


FIG. 1

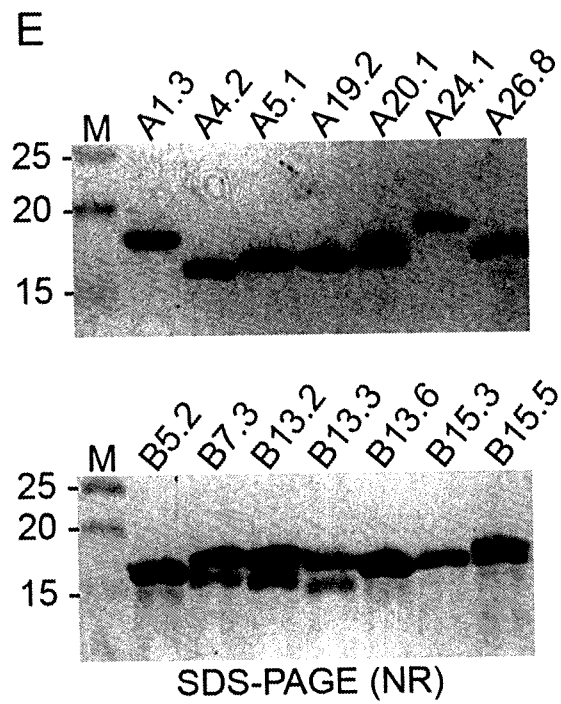
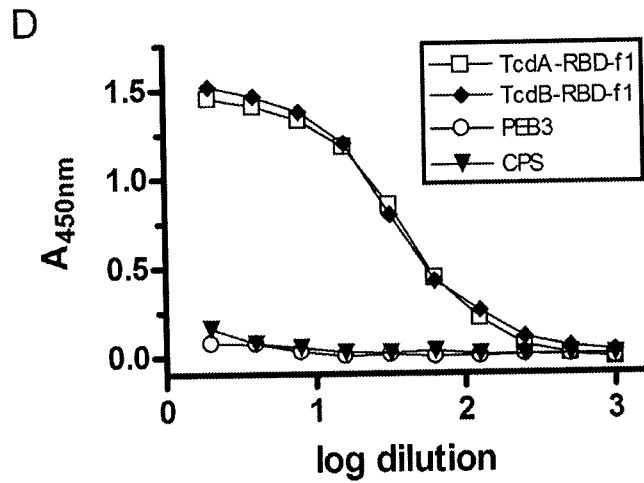


FIG. 1

	FR1	CDR1	FR2	CDR2	
			42 49 50 52		
A1.3	QVKLEESGGGLVQAGGSLRLS	CAASIRSF	SYRNMGWFRQPPGKERE	FVAAITWDGGST	
A4.2	QVKLEESGGGLVQAGGSLRLS	CAASGRTF	NTLSMGWFRQAPGKERE	FVAAVSRSGGST	
A5.1	QVKLEESGGGLVQAGGSLRLS	CAASGRTF	SMYRMGWFRQAPGKERE	FVGIVTRNGSST	
A19.2	QVKLEESGGGLVQPGGSLRLS	CAASGRTL	SSYIVAWFRQAPGKERE	FVAGISRRGGNS	
A20.1	QVQLVESGGGLAQAGGSLRLS	CAASGRTF	SMDPMWFRQPPGKERE	FVAAGSSTGRTT	
A24.1	QVQLVESGGGLVQAGGSLRLS	CAASIRSF	SNRMGWFRQPPGKERE	FVAGISWGGGST	
A26.8	QVKLEESGGGLVQAGGSLRLS	CAASERTF	SRYPVWFRQAPGAERE	FVAIVISSTGTST	
B5.2	QVQLVESGGGLVQPGGSLRLS	CAASGNIFS	INTMGWYRQAPGKQLE	LVAAIT-SGGTT	
B7.3	QVKLEESGGGLVQPGGSLRLS	CAASGRTA	SGYGMGWFRQAPGKERE	FVAAISRSAGT	
B13.2	QVKLEESGGGSVQAGGSLRLS	CAASGRD	FSTLAMGWFRQAPGKERE	FVATINWSGGTT	
B13.3	QVKLEESGGGLVQAGGSLRLS	CSASGSIFS	INDMGWYRRAPGKRRE	LVAAIT-SGGIP	
B13.6	QVKLEESGGGLVQAGGSLRLS	CSASGRTF	SSGVMGWFRQAPGKQRE	LVAAIT-TGGST	
B15.3	QVQLVESGGGSVQAGGSLRLS	CAASG---	LSRYAMWFRQGTGKERE	FVASTNWSSGNT	
B15.5	QVQLVESGGDLVQAGGSLRLS	CAASGSISR	ISTMGWYRQAPGKQRE	LVAITIS-TGGTT	
		FR3		CDR3	
A1.3	RYADSVKGRFTVSRD	NAKKT	VYVYLMNSLKP	EDAAVYYCAAGFGHTLATSSDE----	YDY
A4.2	YYADSVKGRFTISR	DAKNT	VYVYLMNSLKP	EDTAVYYCAAAATKSNTTAYRLS----	FDY
A5.1	YYADSVKGRFTISR	DAKNT	VYVYLMNSLKP	EDTALYYCAATSGSSYLDAAHV----	YDY
A19.2	AYVESVKGRTISR	DAKNT	VYVYLMNSLKP	EDTAVYYCAADGSVAGWGRRSVSVSSYDY	
A20.1	YYADSVKGRFTISR	DAKNT	VYVYLMNSLKP	EDTAVYYCAAAPYGANWYRDE-----	YAY
A24.1	RYADSVKGRFTISR	DAKKT	VYVYLMNSLKP	EDTAVYYCAAIEFGHNIATSSDE----	YDY
A26.8	YYADSVKGRFTISR	DAKVT	VYVYLMNLLKRED	TAVYFCVNSQRTLRQDPNE----	YDY
B5.2	SYTDSVEGRFTISR	DAKNAV	VYVYLMNSLKAED	TAVYYCNTVKVVGGRDLN-----	PDY
B7.3	LNADFVKGRFTISR	DAKNT	VYVYLMNSLKP	EDTAVYYCVARPTKVDRDYATRREM-YN	Y
B13.2	HYADSVKGRFTISR	DAKNT	VYVYLMGSLKP	EDTAVYYCGRSKYAAGALTRAYD----	YNY
B13.3	NYADSVKGRFTISR	DAKNT	GYVYLMNSLKP	EDTAVYYCAAQFGTVAAALRRHE---	YDY
B13.6	SYTDSVKGRFTISR	DAKNT	VYVYLMNSLKP	EDTAVYYCNSVAVVGGVIKS-----	PDY
B15.3	PYADSVKGRFTISR	DAKNT	VYVYLMNSLKP	GDTAIYYCAARKLDVPSRYSQH----	YDY
B15.5	NYAESVKGRFTVSR	DAKNT	MYVYLMNSLKP	EDTAVYYCAAGWKVVRGSLE-----	YEY
		FR4			
A1.3	WGQGTQVTVSS				
A4.2	WGQGTQVTVSS				
A5.1	WGQGTQVTVSS				
A19.2	WGQGTQVTVSS				
A20.1	WGQGTQVTVSS				
A24.1	WGQGTQVTVSS				
A26.8	WGQGTQVTVSS				
B5.2	WGQGTQVTVSS				
B7.3	WGQGTQVTVSS				
B13.2	WGQGTQVTVSS				
B13.3	WGQGTQVTVSS				
B13.6	WGQGTQVTVSS				
B15.3	WGQGTQVTVSS				
B15.5	SGQGTQVTVSS				

FIG. 2

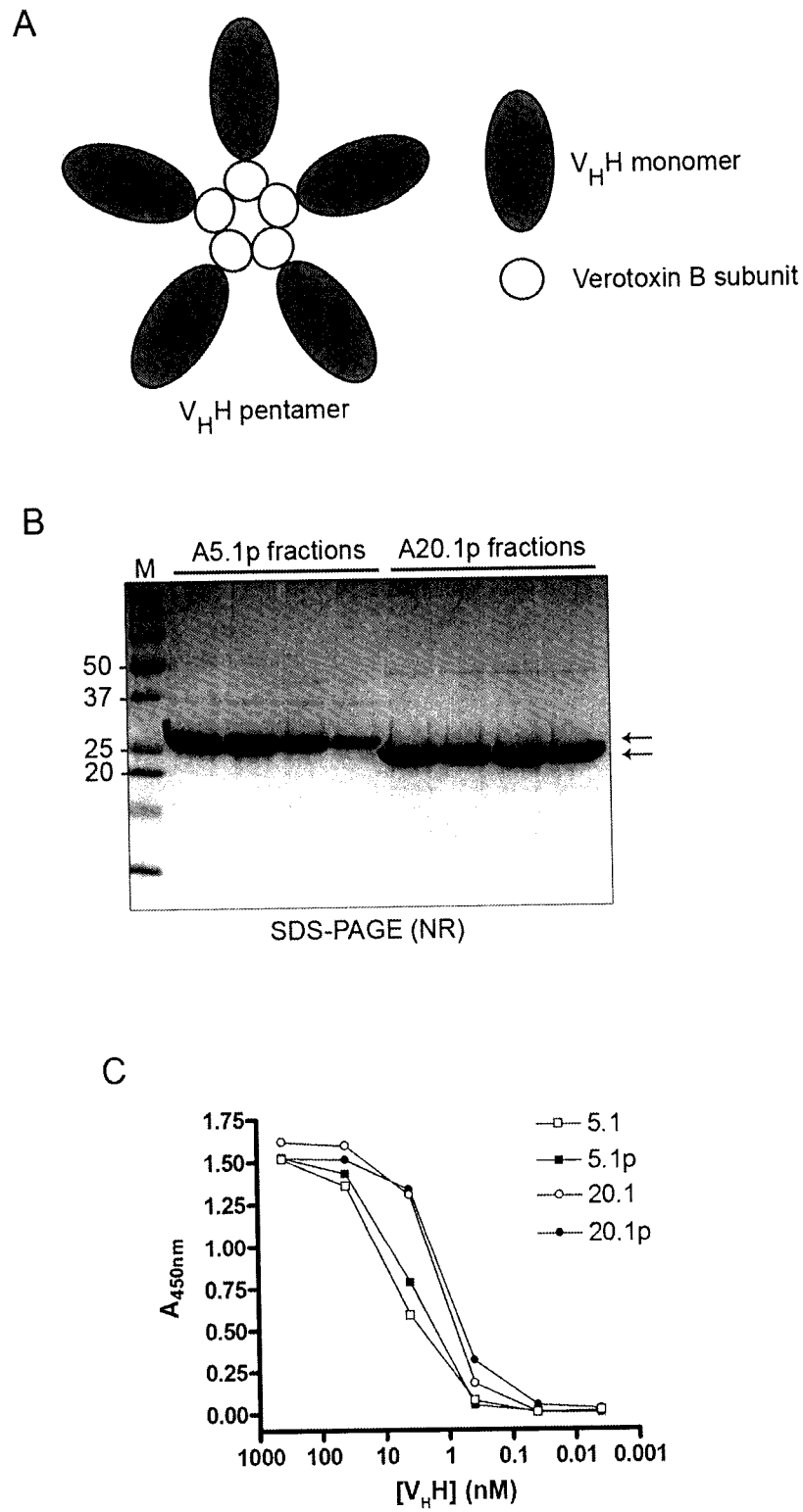


FIG. 3

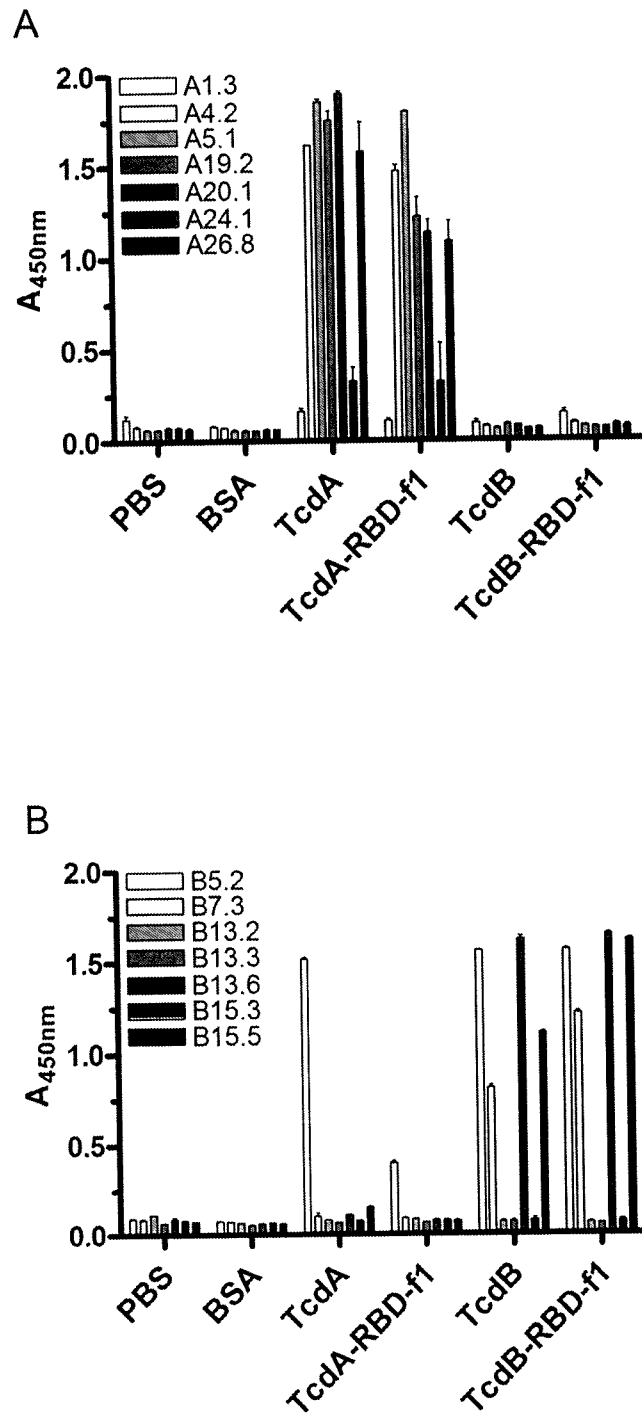


FIG. 4

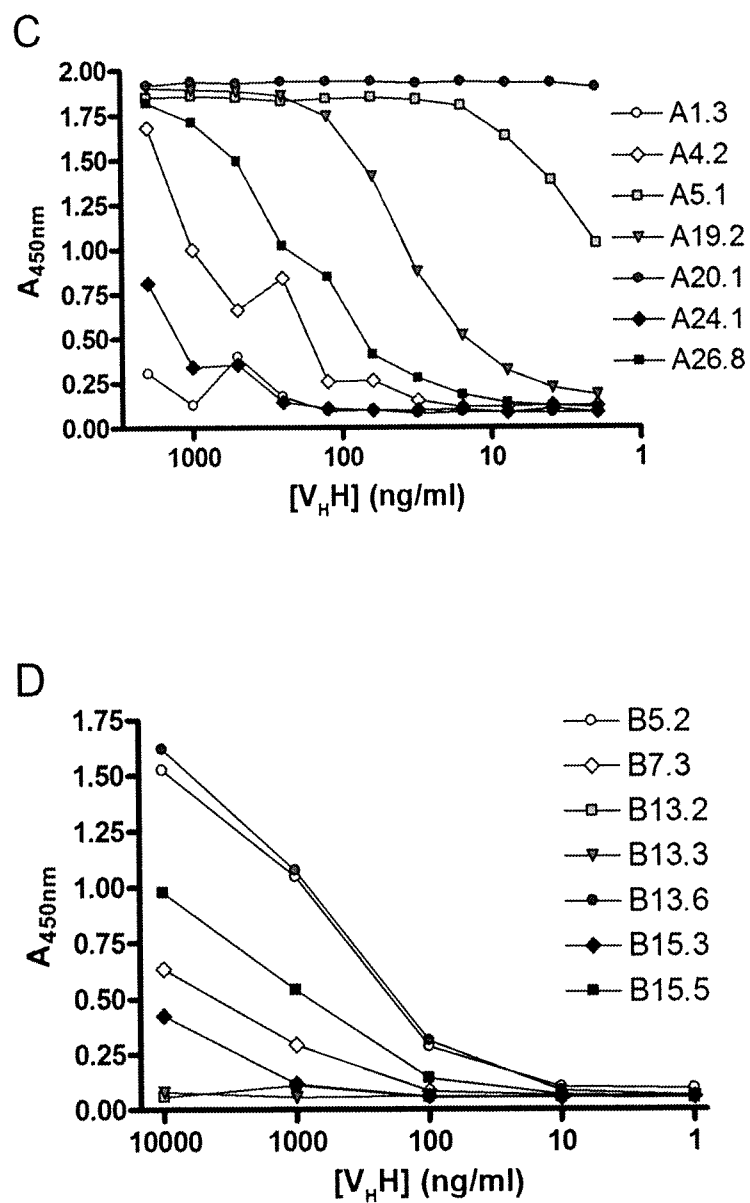


FIG. 4

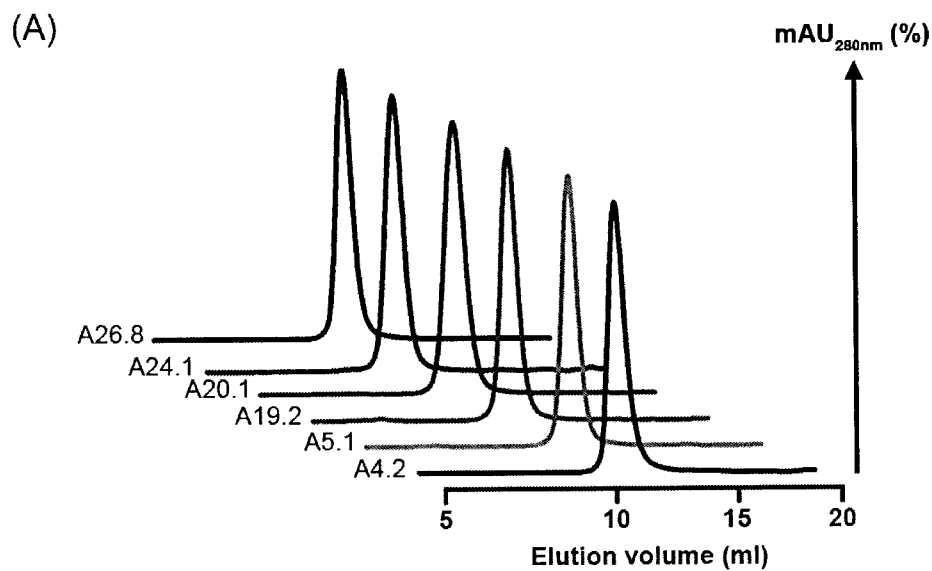


FIG. 5

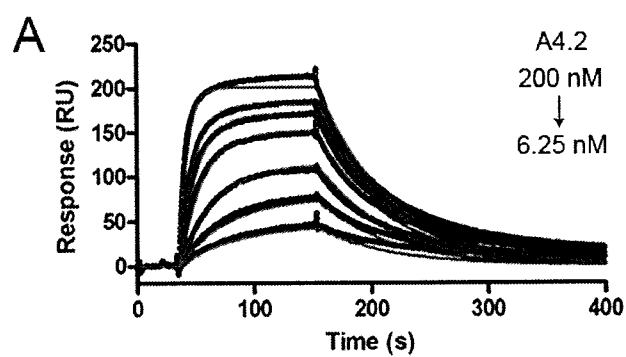


FIG. 6

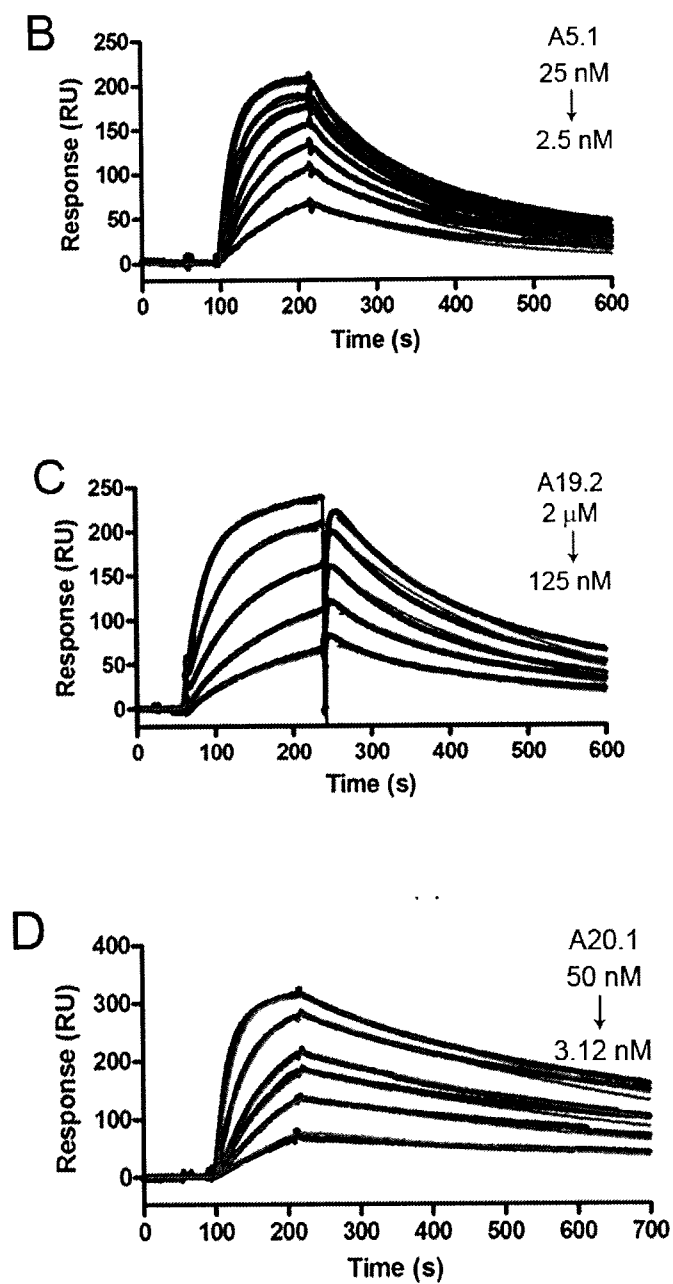


FIG. 6

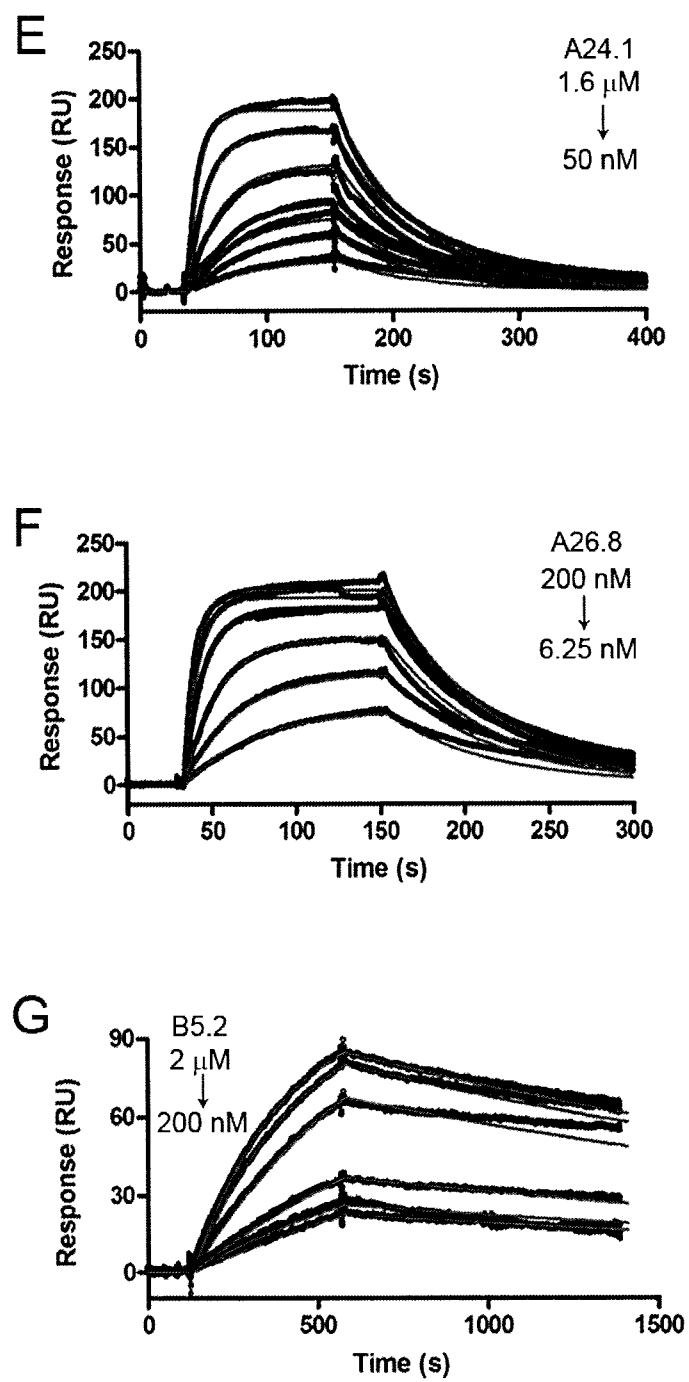


FIG. 6

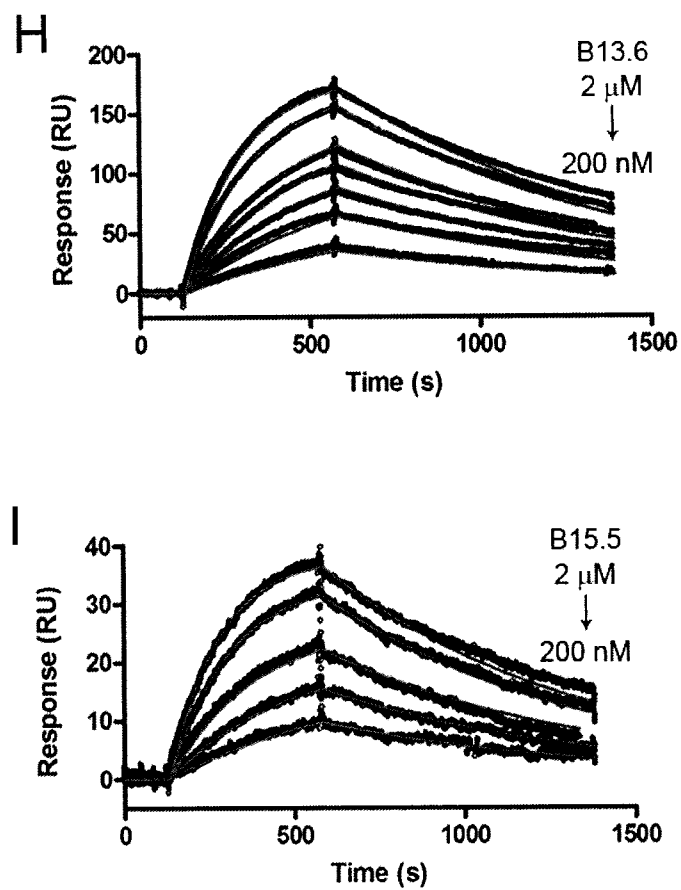


FIG. 6

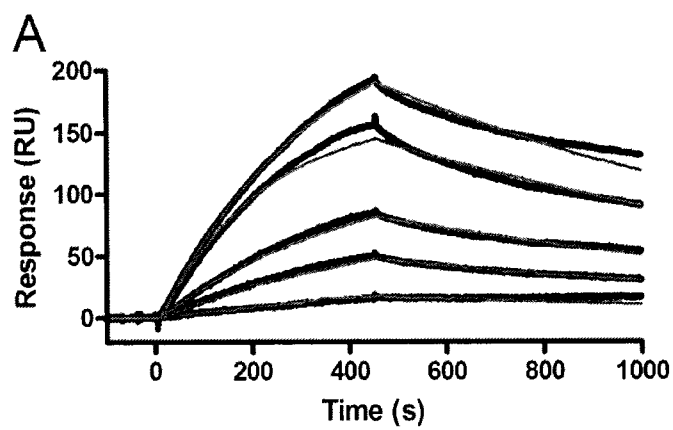


FIG. 7

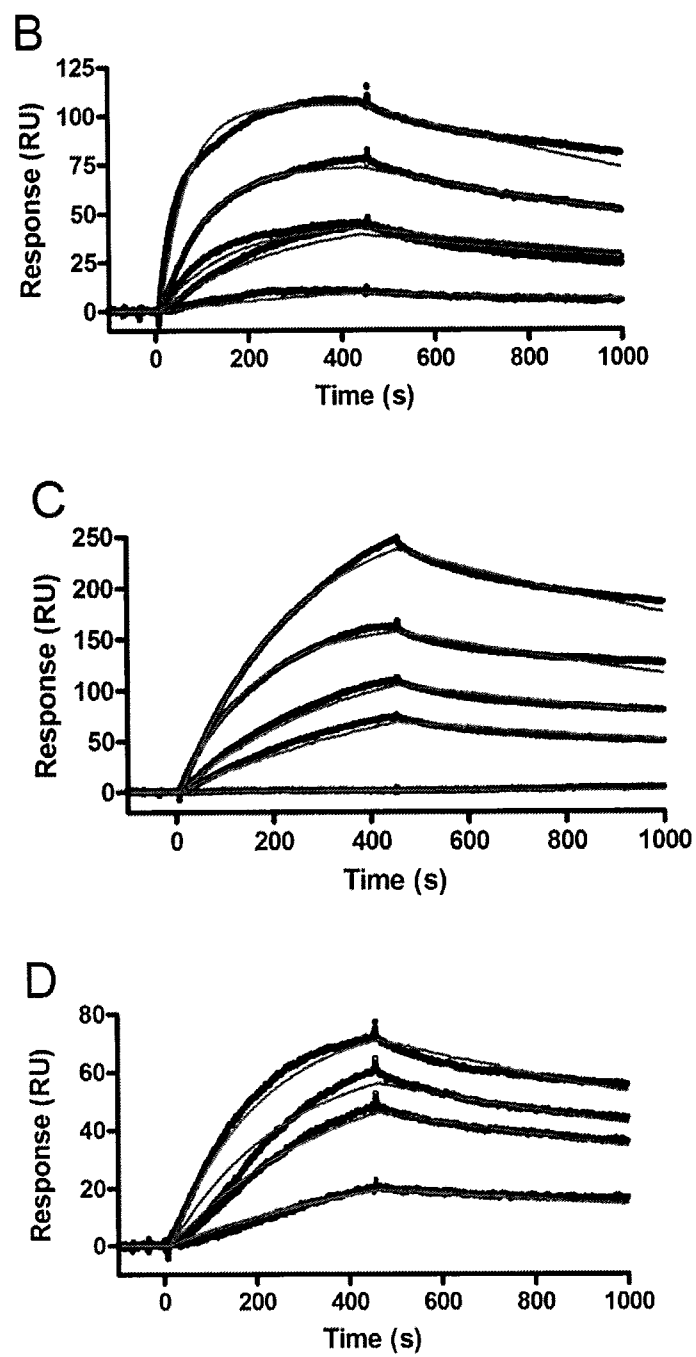


FIG. 7

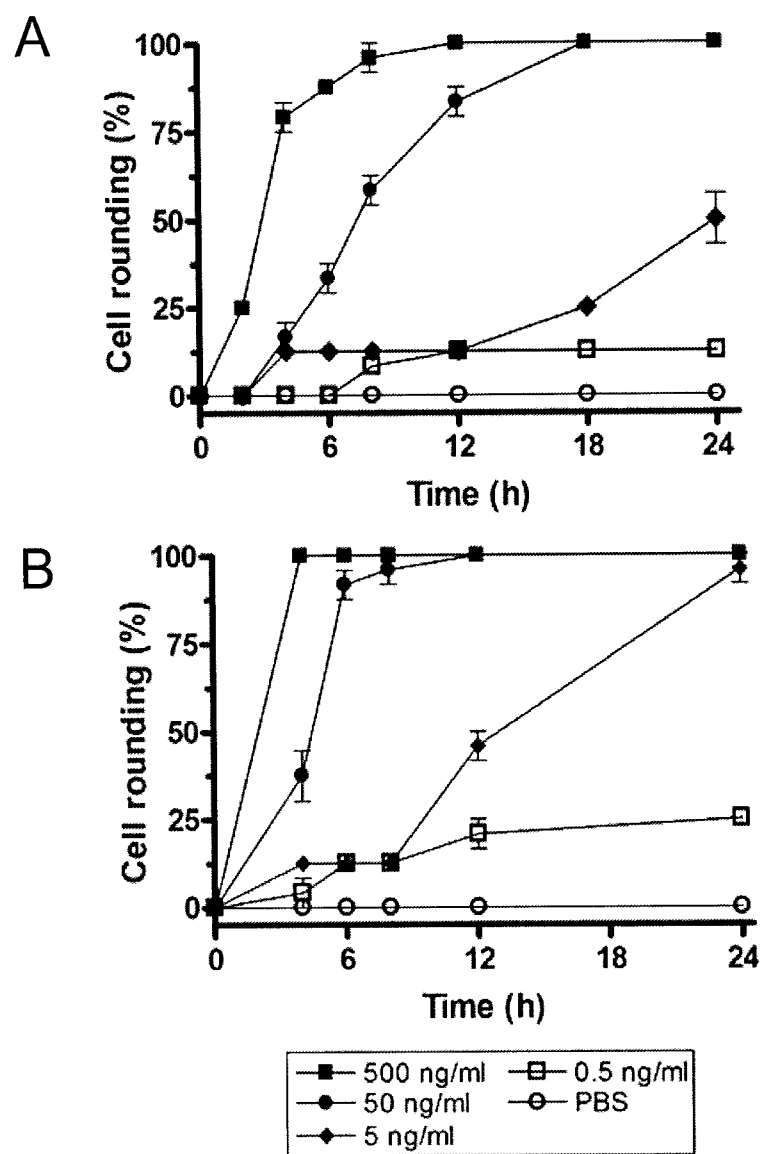


FIG. 8

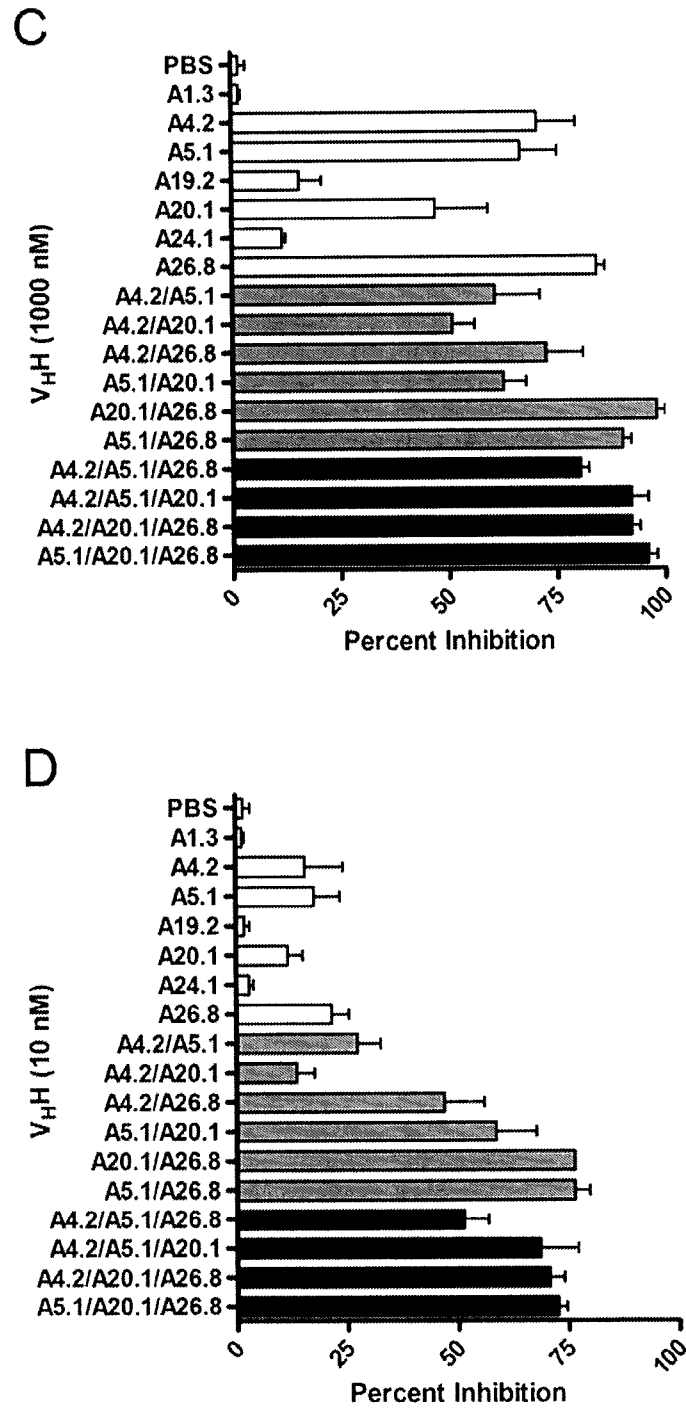


FIG. 8

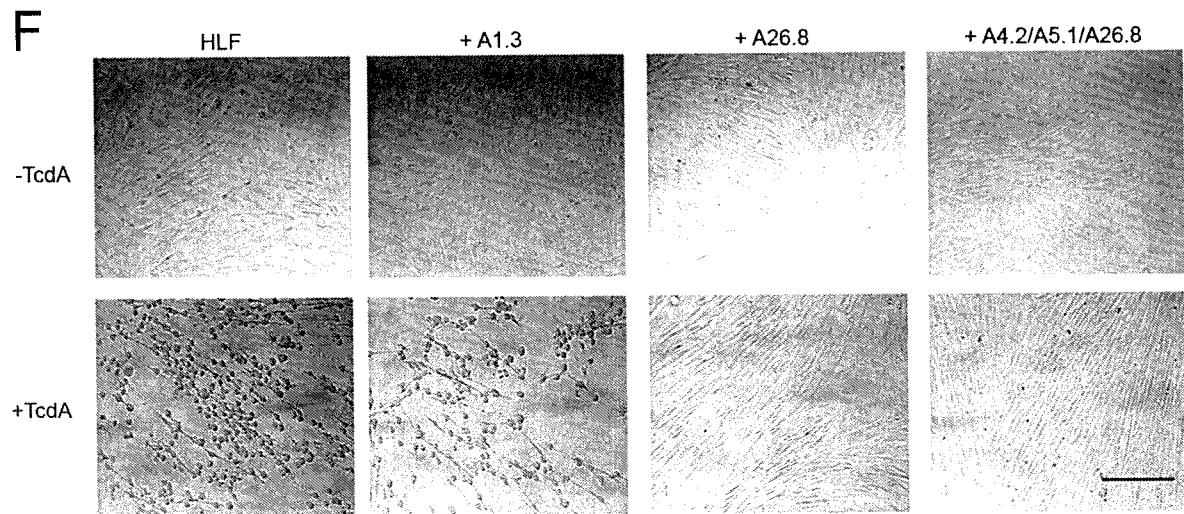
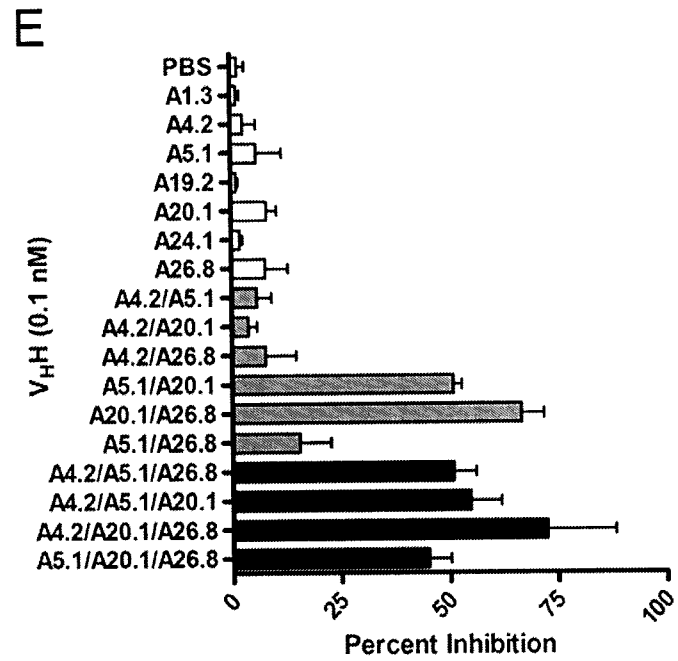


FIG. 8

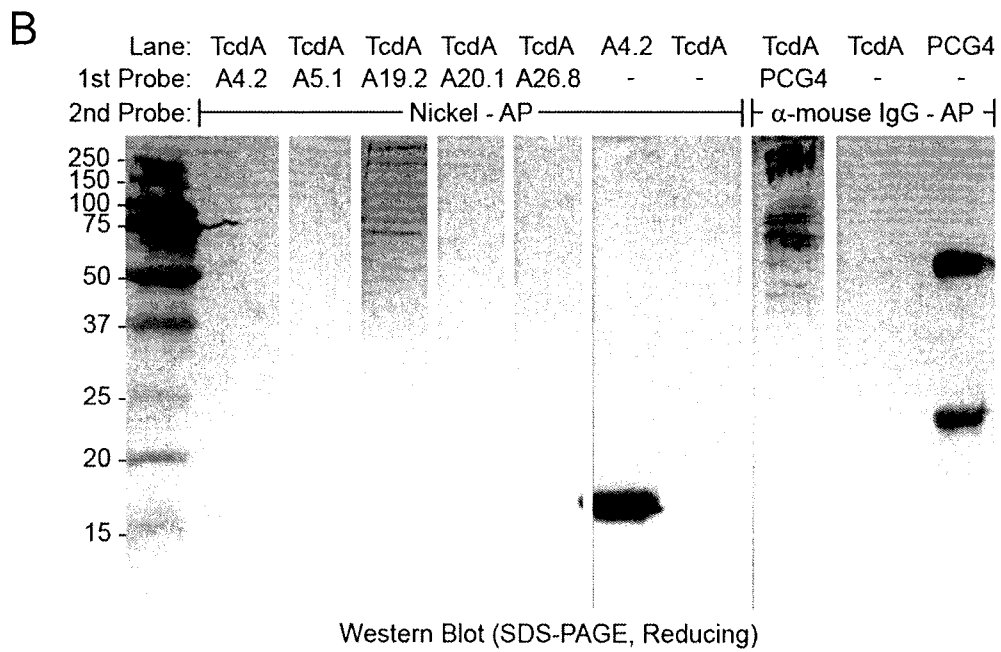
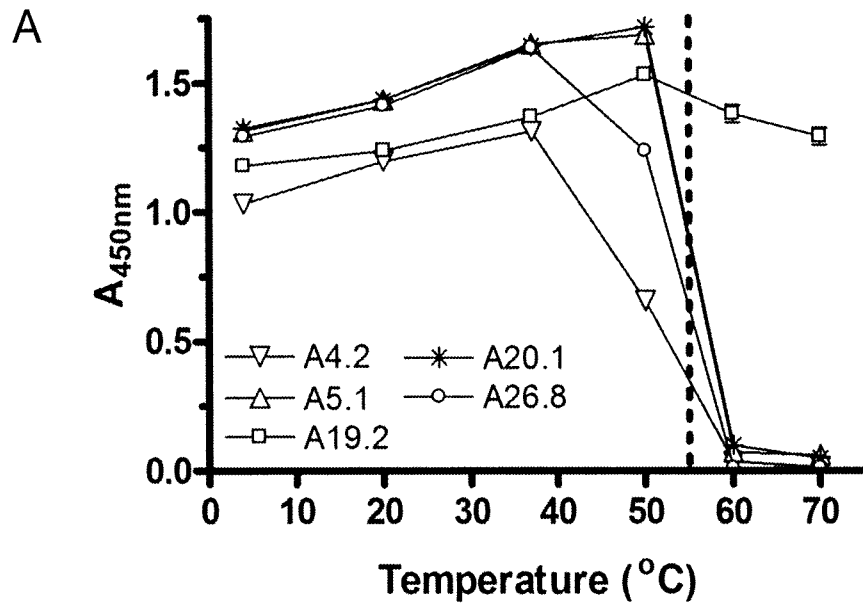


FIG. 9

C

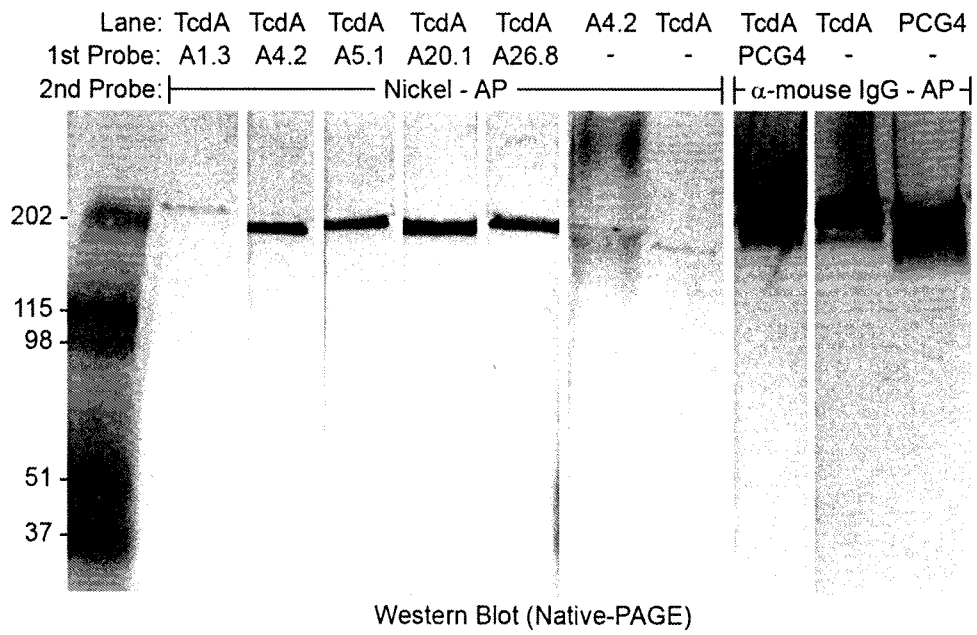


FIG. 9

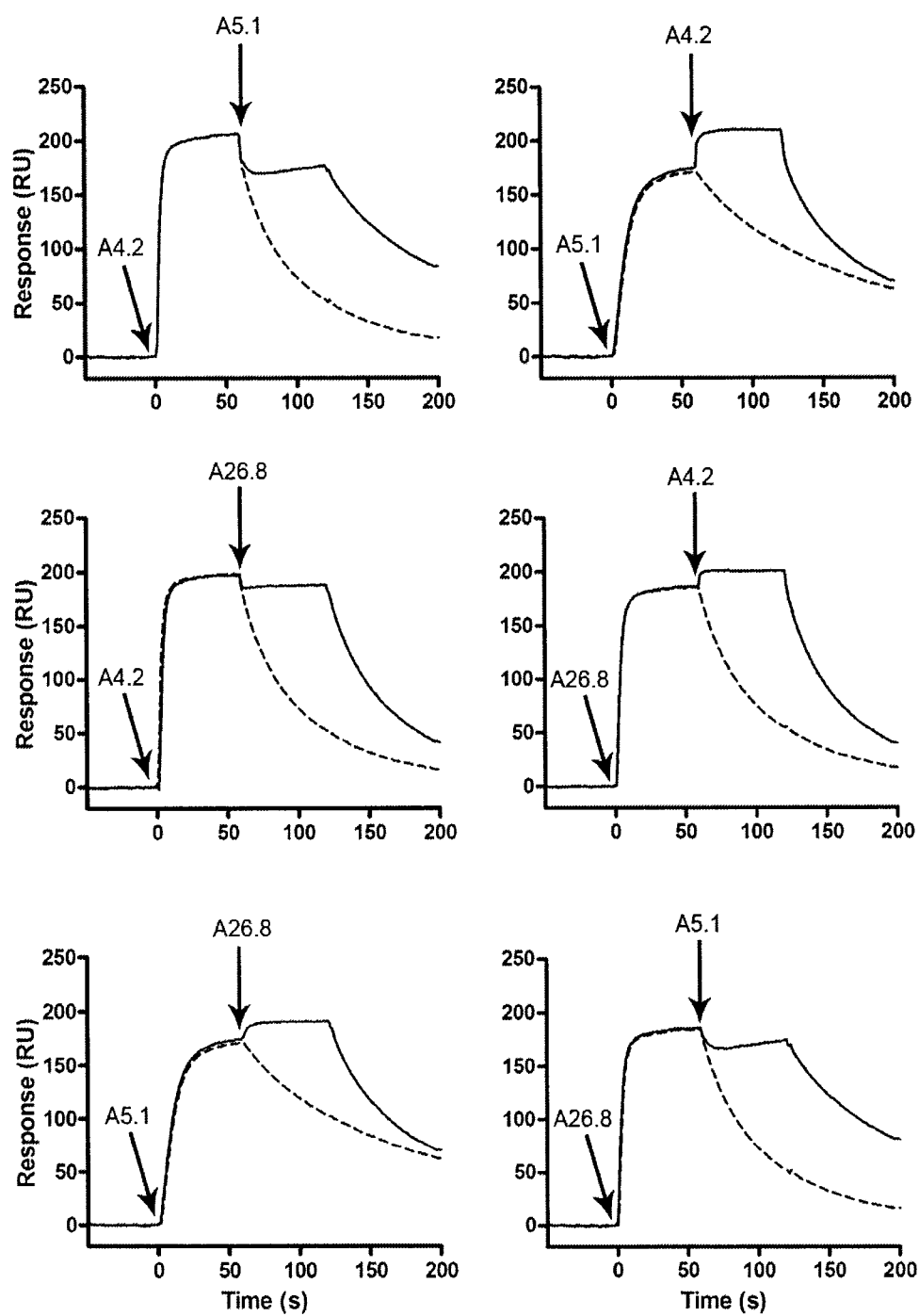


FIG. 10

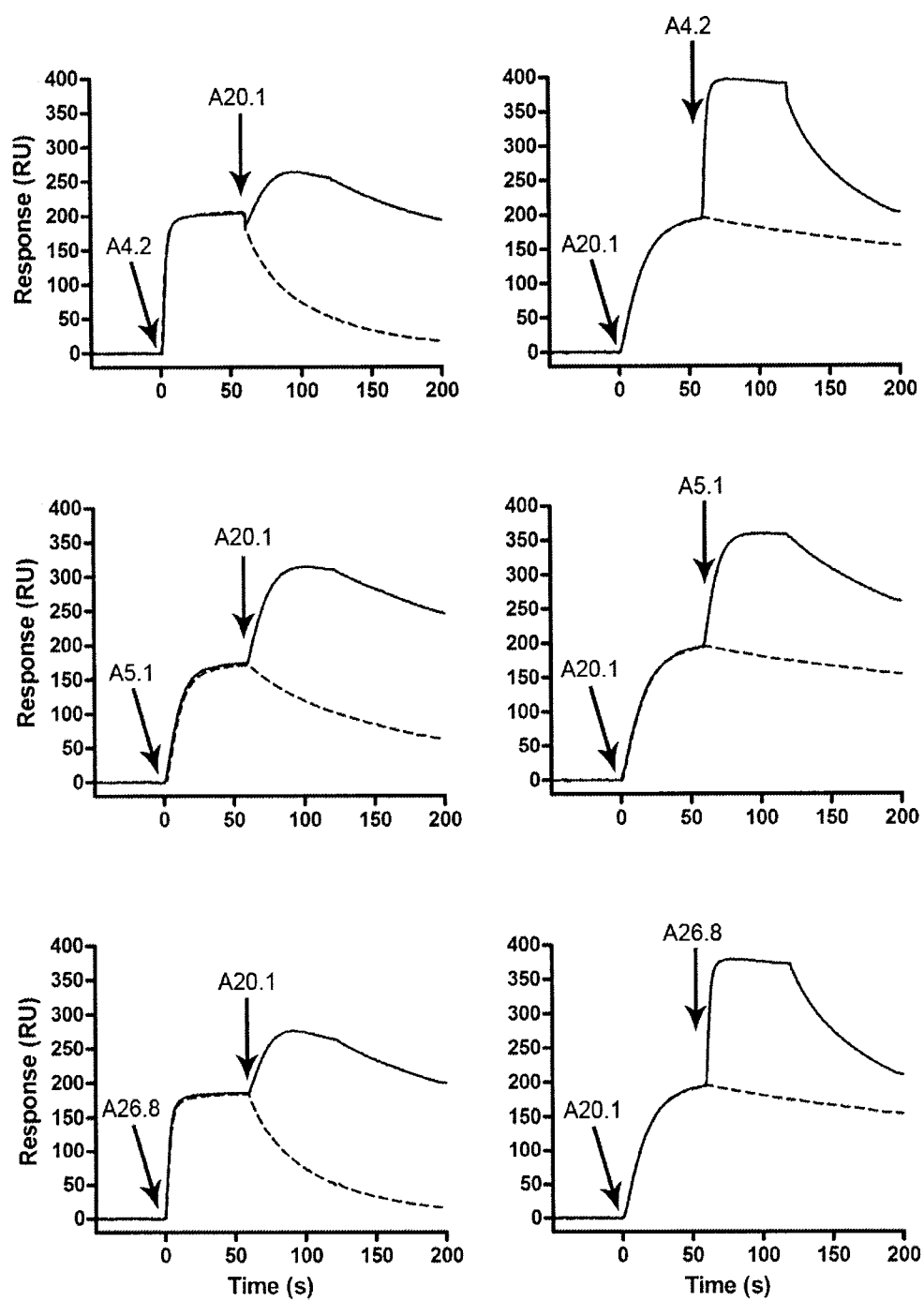


FIG. 10

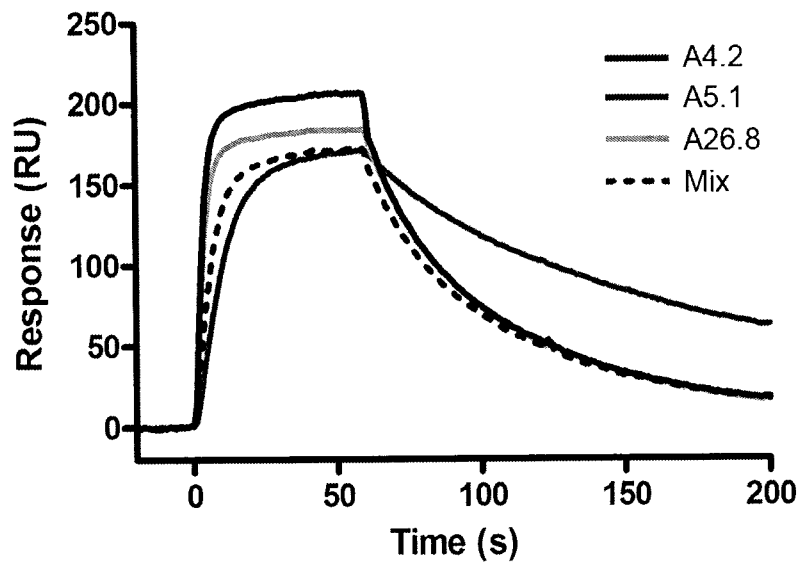


FIG. 11

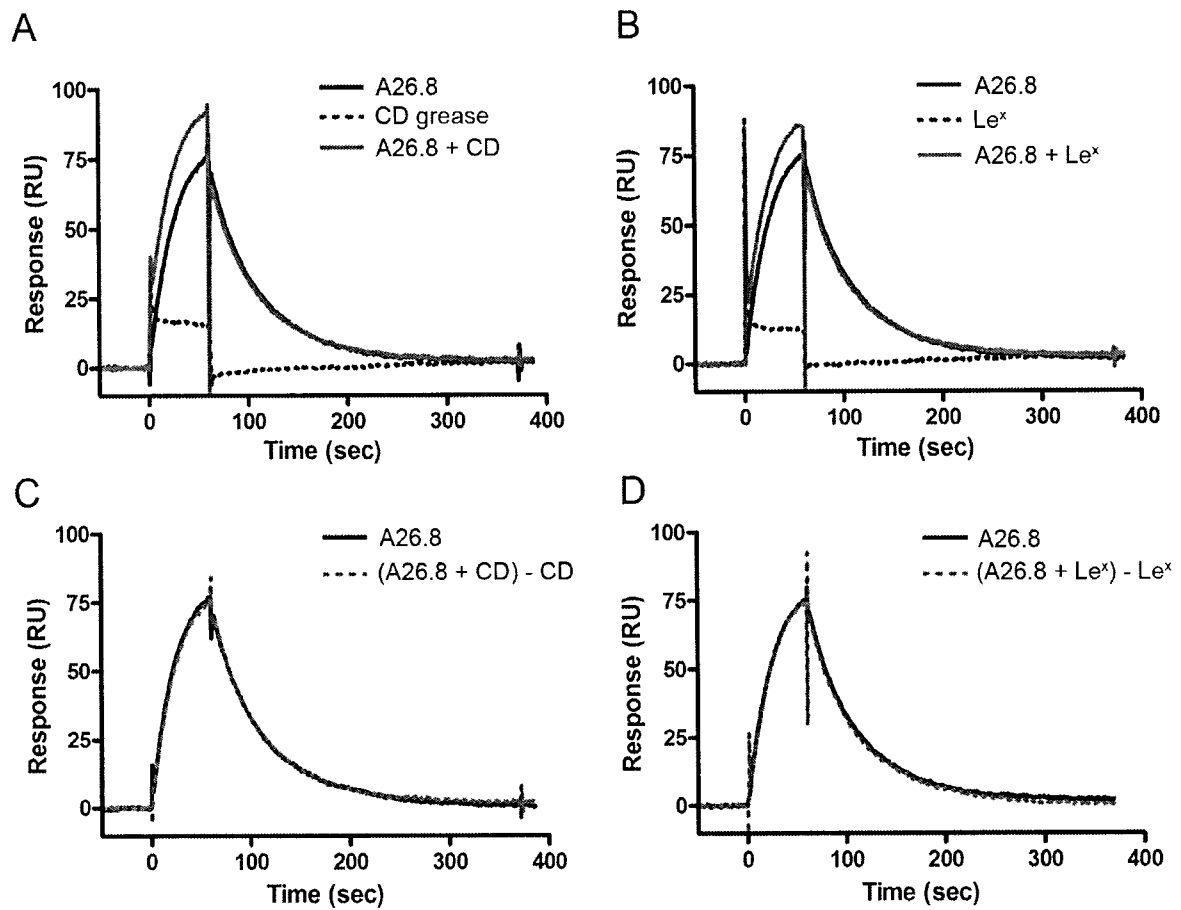


FIG. 12

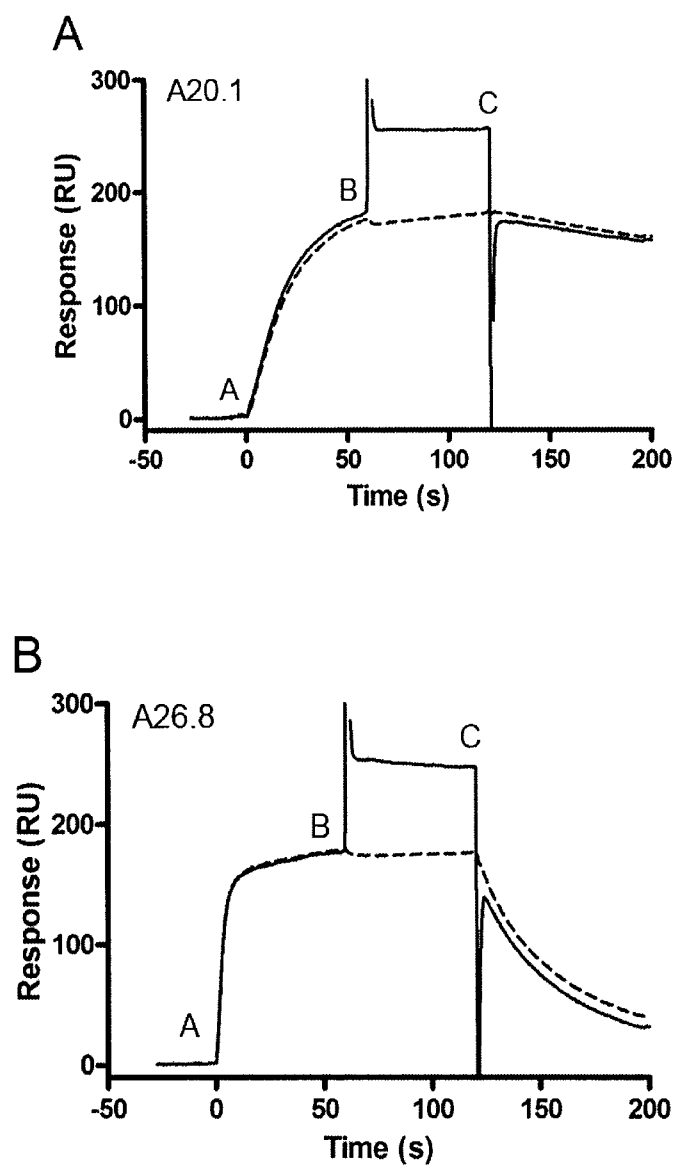


FIG. 13

WT	FR1	CDR1	FR2	*	CDR2	*	FR3	CDR3	FR4
A4.2	QVKLEESGGGLVQAGGSLRLSCAASGRTFNTILSMGWFRQAPGKEREFVCAVSRSGGSTYYADSVKGRFTISRDNAKNTVYLQMNLSLKPEDTAVYYCAAAATKSNNTAYRLS---								FDYWGQGGTQVTVSS
A5.1	QVKLEESGGGLVQAGGSLRLSCAASGRTFNTILSMGWFRQAPGKEREFVCAVSRSGGSTYYADSVKGRFTISRDNAKNTVYLQMNLSLKPEDTALYCAATSGSSYLDAAHV---								YDYWGQGGTQVTVSS
A19.2	QVKLEESGGGLVQAGGSLRLSCAASGRTLSSTSYIVAFWRQAPGKEREFVCAVSRSGGSTYYADSVKGRFTISRDNAKNTVYLQMNLSLKPEDTAVYYCAADGSVAGWGRRSVSVSSYDYWGQGGTQVTVSS								
A20.1	QVQLVESGGGLAQAGGSLRLSCAASGRTFSMDPMAWFRQPPGKEREFVCAVSRSGGSTGRITYYADSVKGRFTISRDNAKNTVYLQMNLSLKPEDTAVYYCAAAAPYGANWYRDE---								YAYWGQGGTQVTVSS
A24.1	QVQLVESGGGLVQAGGSLRLSCAASIRSFNRNMGWFRQPPGKEREFVCAVSRSGGSTYYADSVKGRFTISRDNAKNTVYLQMNLSLKPEDTAVYYCAAEFGHNIAATSSDE---								YDYWGQGGTQVTVSS
A26.8	QVKLEESGGGLVQAGGSLRLSCAASERTFSRYPVANFRQAPGAEREFFVAVISSITGTSTYYADSVKGRFTISRDNAKNTVYLQMNLSLKPEDTAVYFCVAVNSQRTLRQDPNE---								YDYWGQGGTQVTVSS
Mut									
A4.2m	QVKLEESGGGLVQAGGSLRLSCAASGRTFNTILSMGWFRQAPGKEREFVCAVSRSGGSTYYADSVKGRFTCSRDNAKNTVYLQMNLSLKPEDTAVYYCAAAATKSNNTAYRLS---								FDYWGQGGTQVTVSS
A5.1m	QVKLEESGGGLVQAGGSLRLSCAASGRTFNTILSMGWFRQAPGKEREFVCAVSRSGGSTYYADSVKGRFTCSRDNAKNTVYLQMNLSLKPEDTALYCAATSGSSYLDAAHV---								YDYWGQGGTQVTVSS
A19.2m	QVKLEESGGGLVQAGGSLRLSCAASGRTLSSTSYIVAFWRQAPGKEREFVCAVSRSGGSTYYADSVKGRFTCSRDNAKNTVYLQMNLSLKPEDTAVYYCAADGSVAGWGRRSVSVSSYDYWGQGGTQVTVSS								
A20.1m	QVQLVESGGGLAQAGGSLRLSCAASGRTFSMDPMAWFRQPPGKEREFVCAVSRSGGSTGRITYYADSVKGRFTCSRDNAKNTVYLQMNLSLKPEDTAVYYCAAAAPYGANWYRDE---								YAYWGQGGTQVTVSS
A24.1m	QVQLVESGGGLVQAGGSLRLSCAASIRSFNRNMGWFRQPPGKEREFVCAVSRSGGSTYYADSVKGRFTCSRDNAKNTVYLQMNLSLKPEDTAVYYCAAEFGHNIAATSSDE---								YDYWGQGGTQVTVSS
A26.8m	QVKLEESGGGLVQAGGSLRLSCAASERTFSRYPVANFRQAPGAEREFFVAVISSITGTSTYYADSVKGRFTCSRDNAKNTVYLQMNLSLKPEDTAVYFCVAVNSQRTLRQDPNE---								YDYWGQGGTQVTVSS

FIG. 14

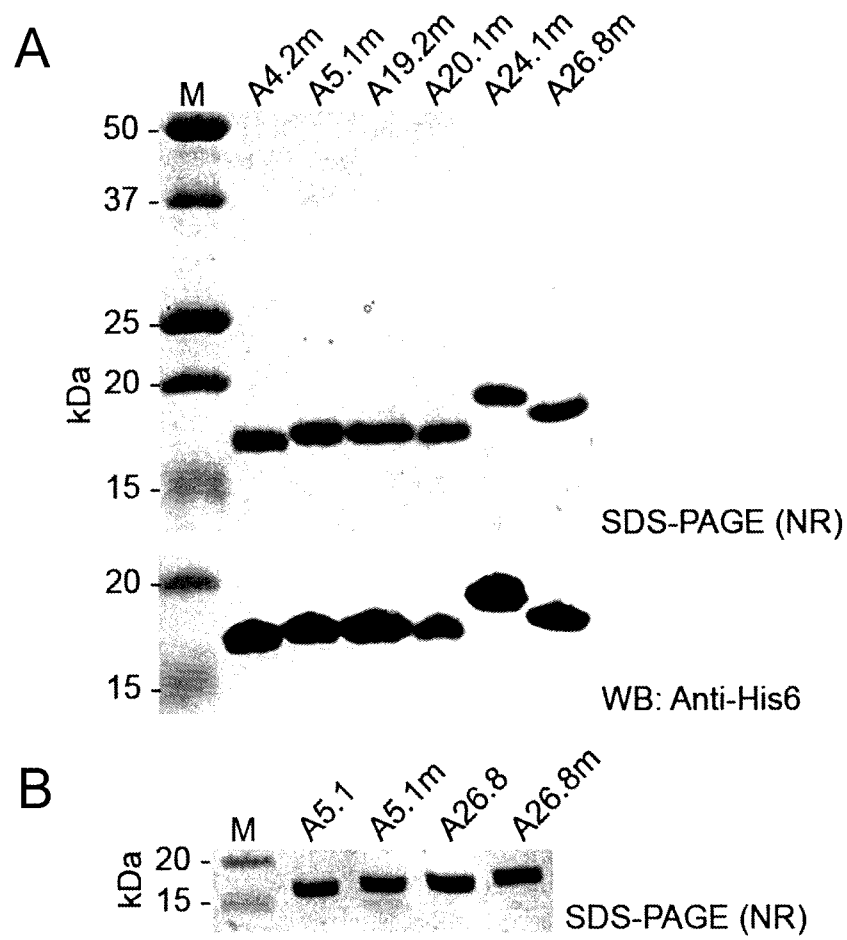


FIG. 15

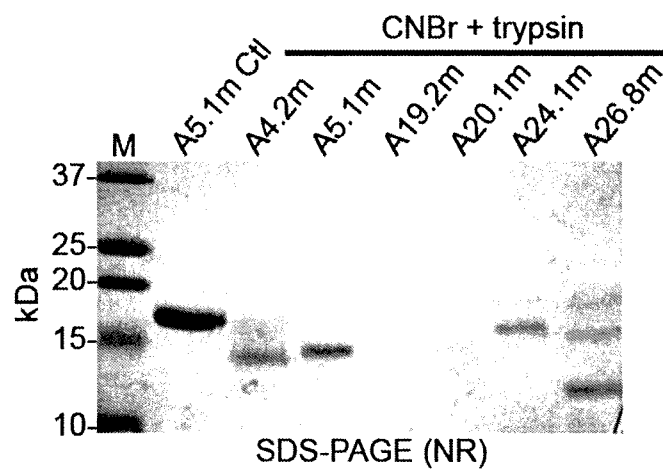


FIG. 16A

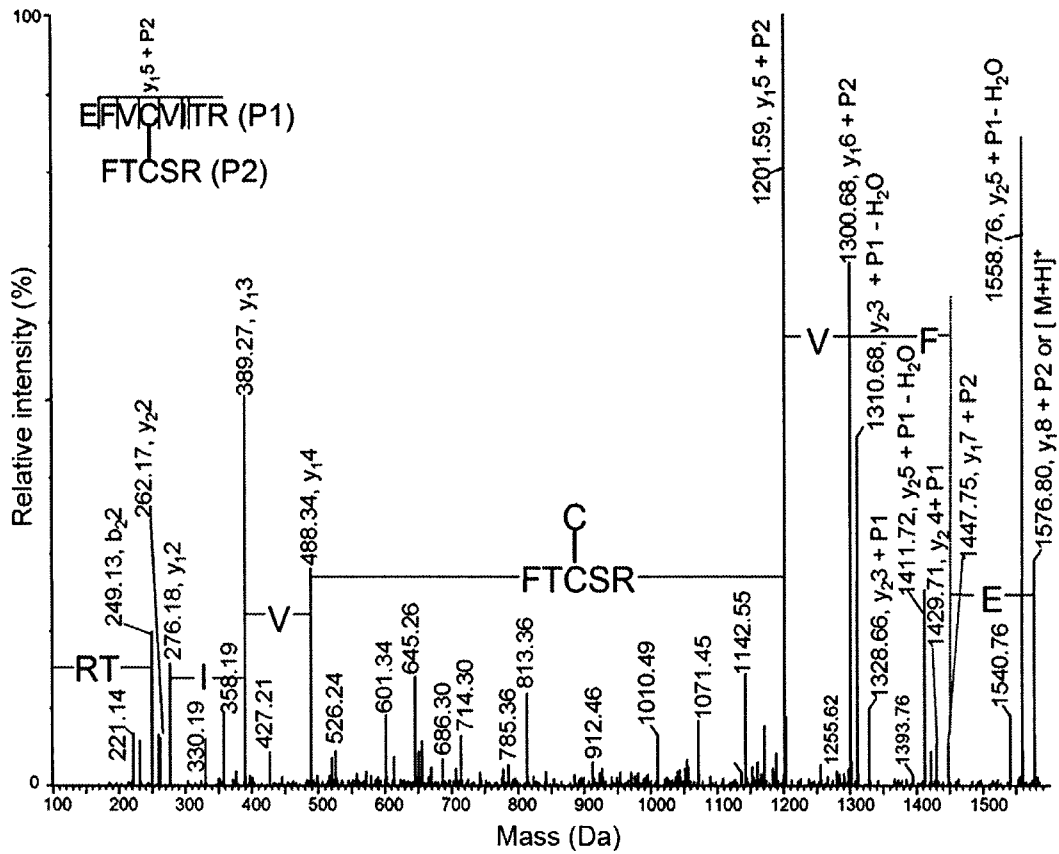


FIG. 16B

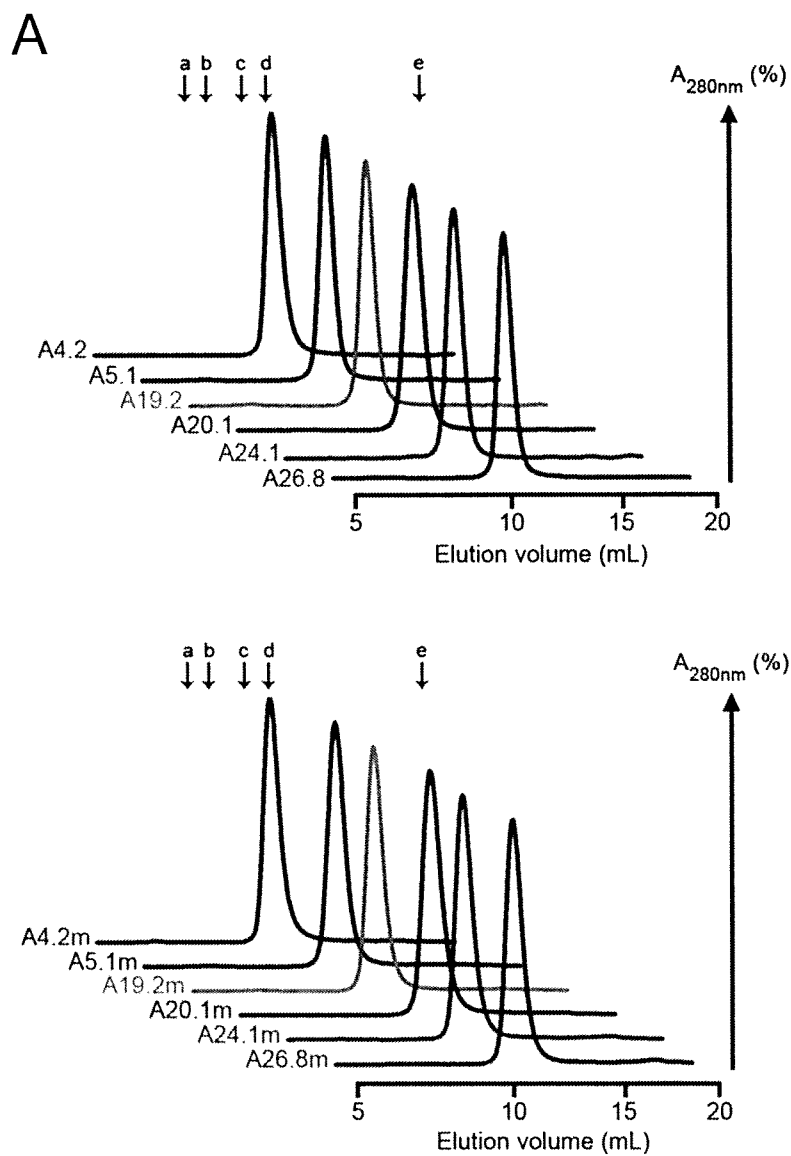


FIG. 17

(B)

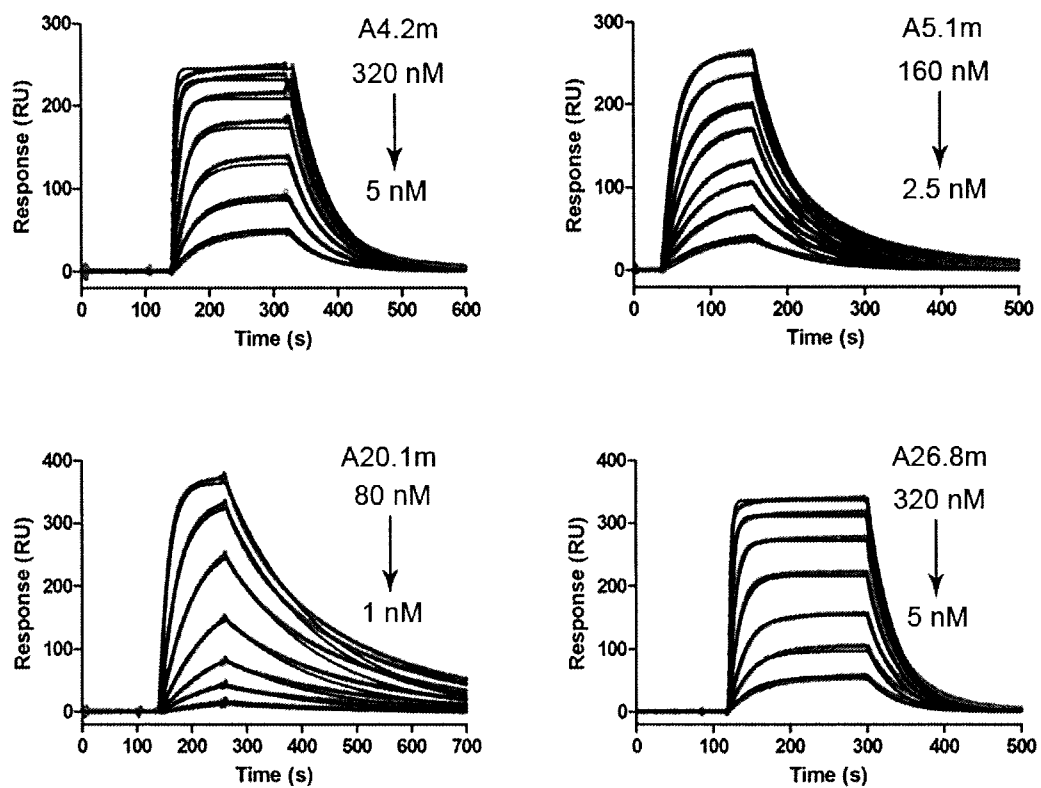


FIG. 17

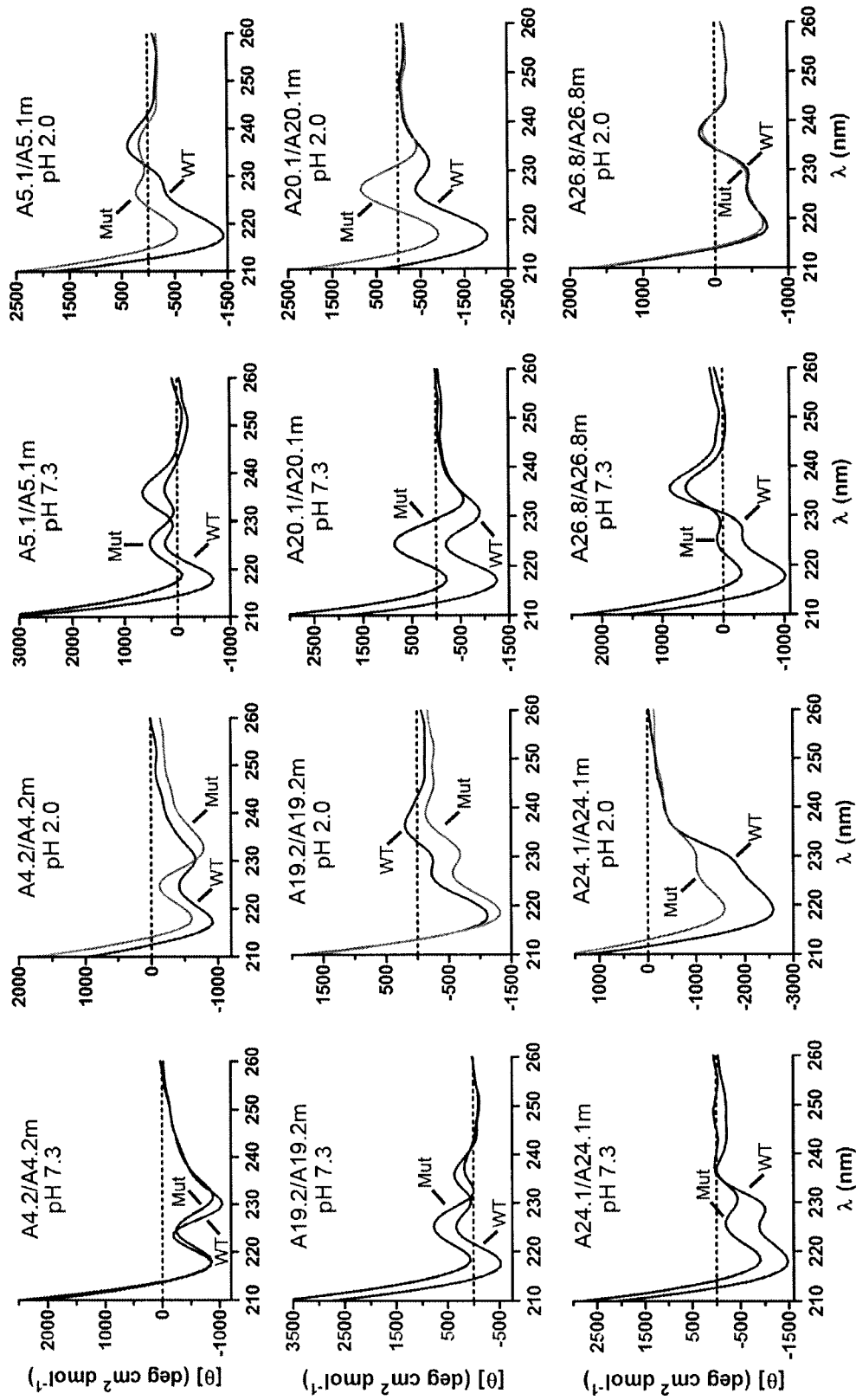


FIG. 18

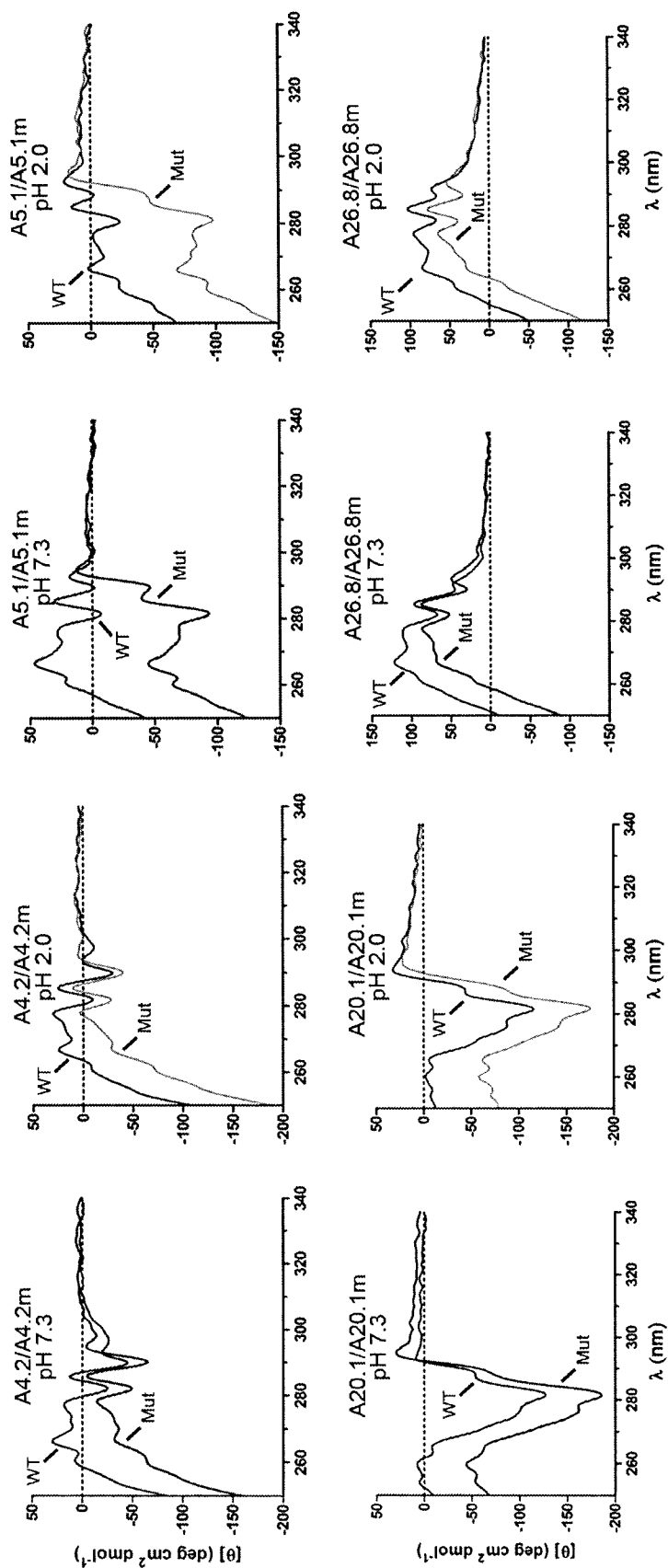


FIG. 19

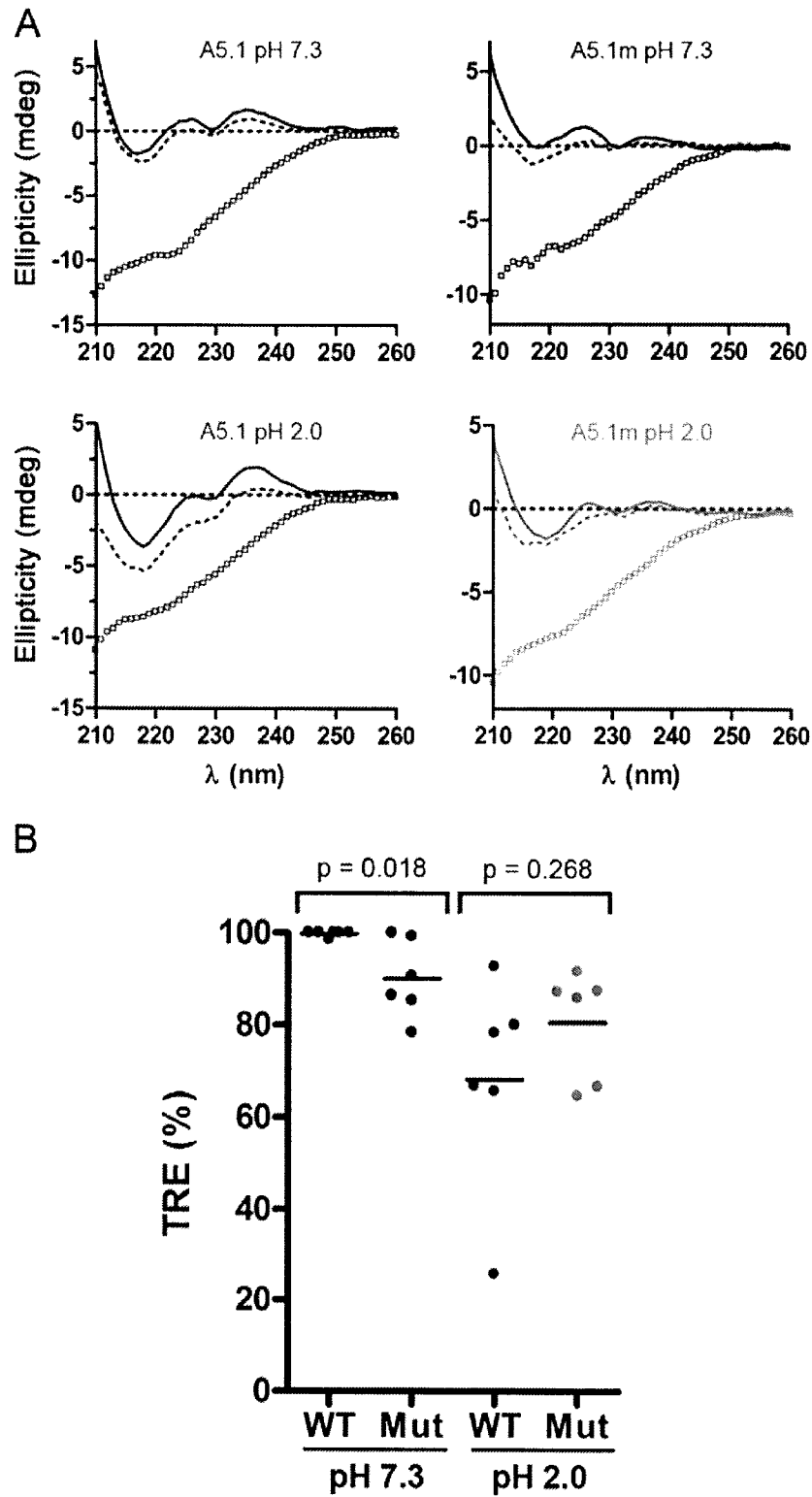


FIG. 20

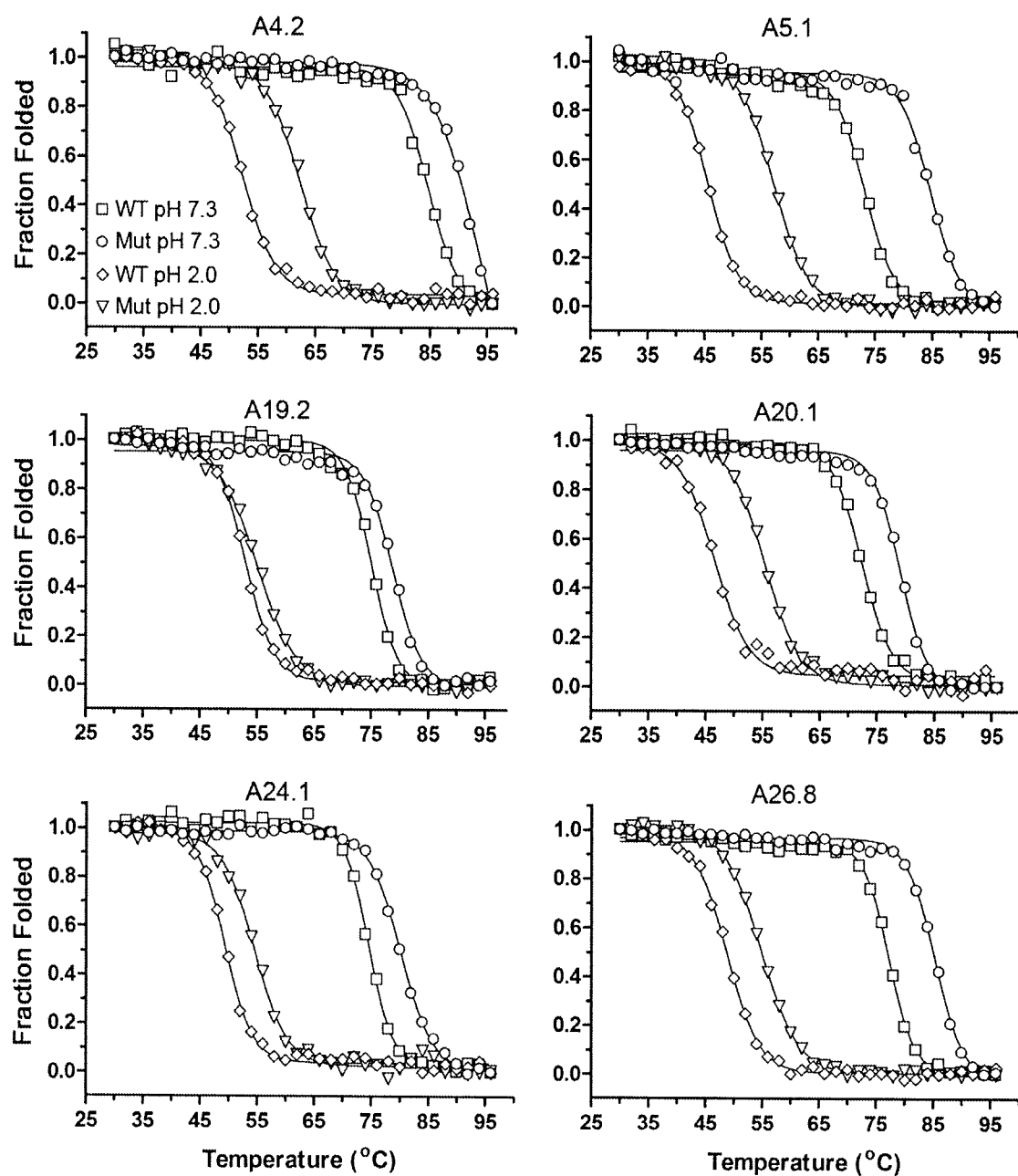


FIG. 21A

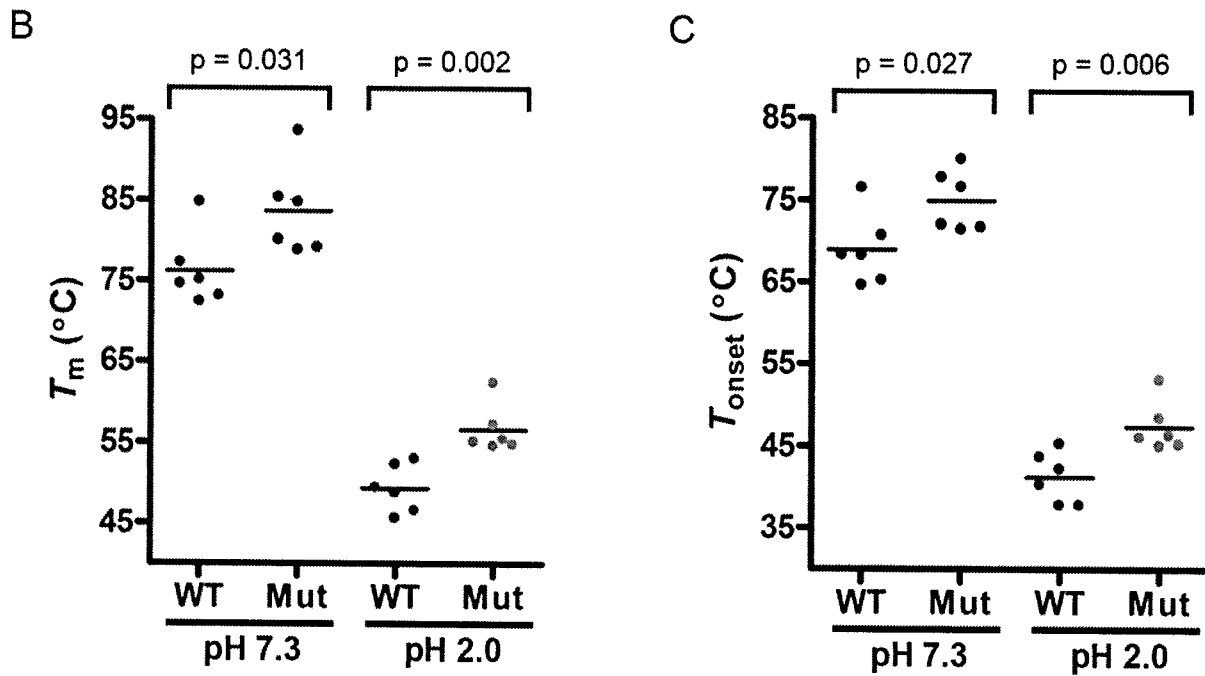


FIG. 21

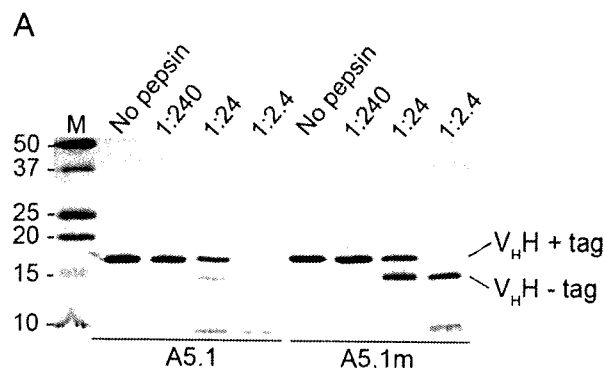


FIG. 22

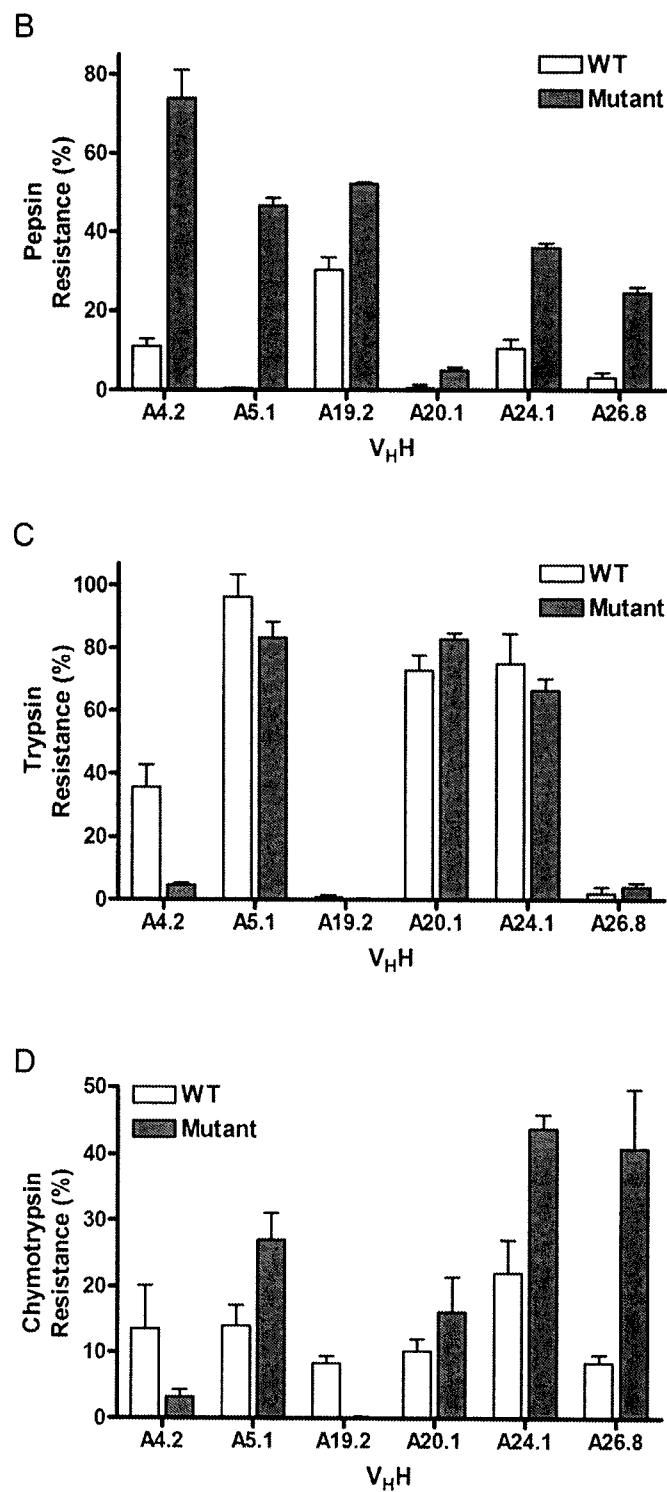
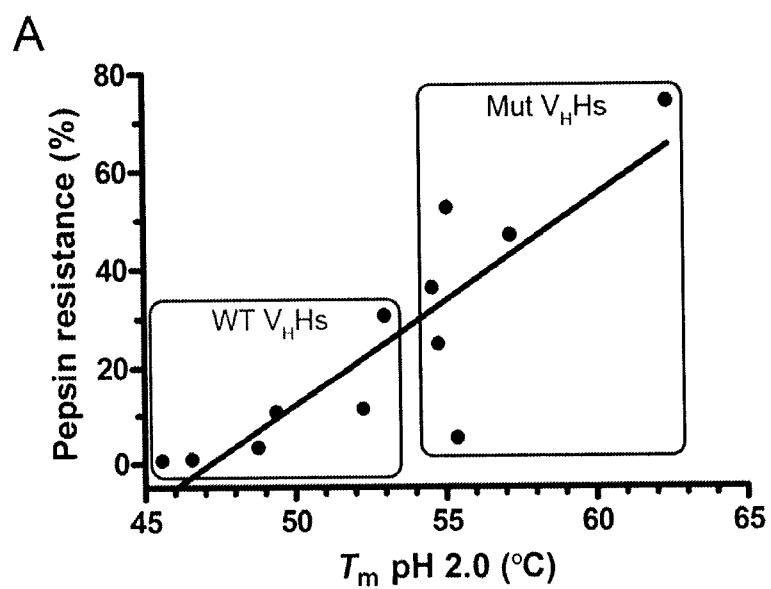
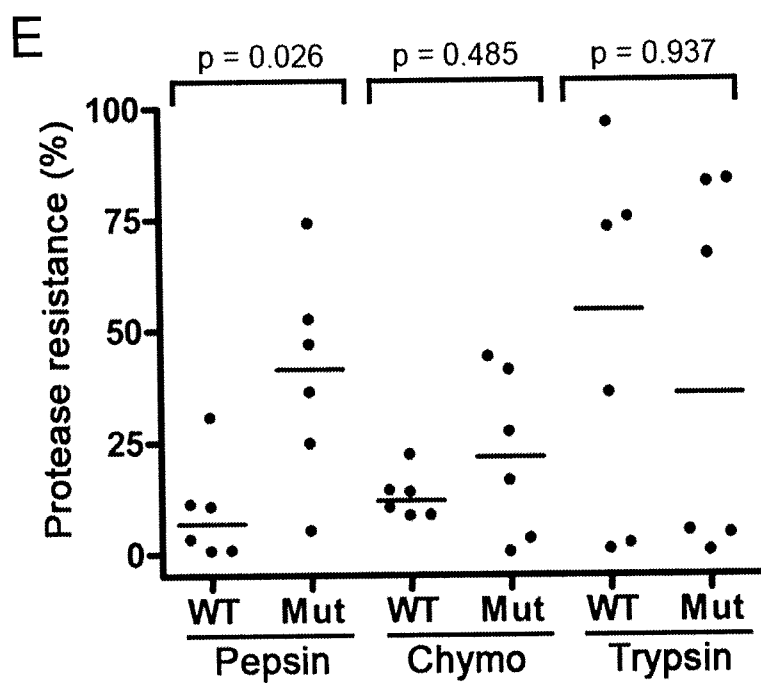


FIG. 22



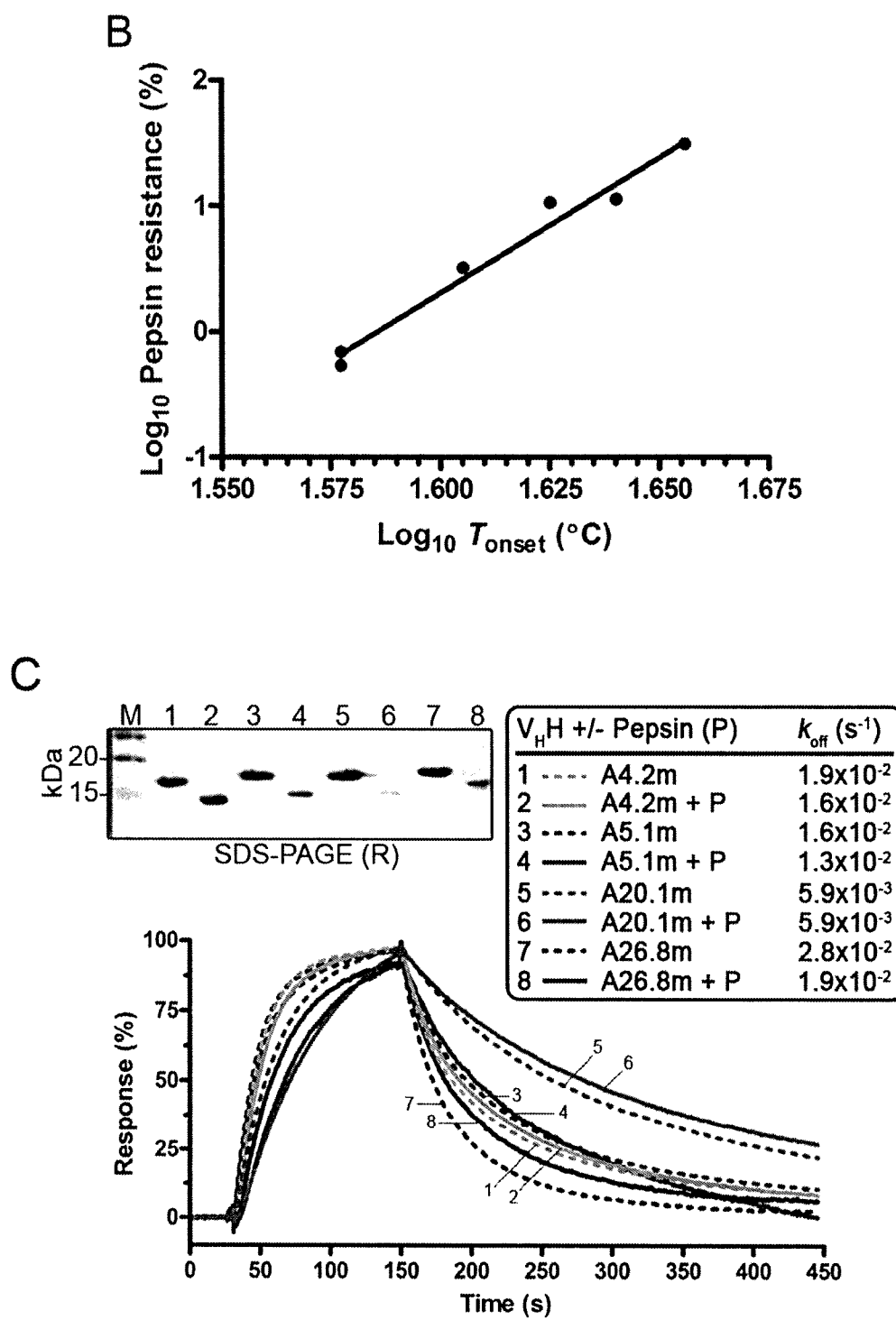


FIG. 23

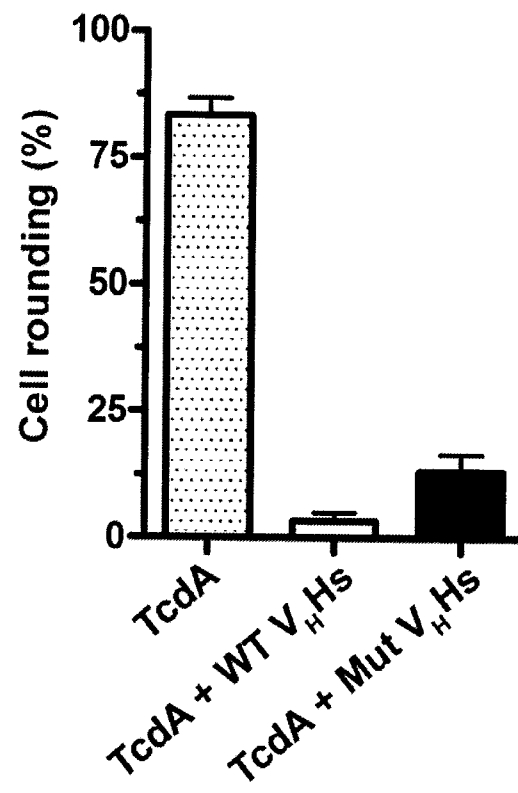


FIG. 24

