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Probes for IgA1 proteases

Versatile substrates and probes for IgA1 protease activity

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Bacterial meningitis is a severe infectious disease with high mortality. Gram-positive and gram-negative bacteria that cause meningitis secrete immunoglobulin A1 (IgA1) proteases that assist in mucosal colonization, invasion, and immune evasion. IgA1 proteases have unique selectivity, with few reported substrates other than IgA1 derived from human tissues. Here we describe the design, characterization, and application of peptide substrates for diverse IgA1 proteases from Neisseria, Haemophilus *and* Streptococcus *bacteria. IgA1 proteases from diverse strains show unexpected selectivity profiles among peptide substrates derived from autoproteolytic sites. A fluorescence probe derived from one of these peptides is used to quantitate IgA1 protease activity in buffer and in human cerebrospinal fluid, and is able to detect less than 1 μg/mL of recombinant* H. influenzae *type 1 IgA1 protease. We also use the probe to establish the first high-throughput screen for IgA1 protease inhibitors. This work provides tools that will help investigate the roles of IgA1 proteases in bacterial colonization, immune evasion, and infection.*

Keywords: peptide sensors · immunoglobulin A · proteases · bacterial meningitis · high-throughput screening

Introduction

Bacterial meningitis is a severe, often fatal infectious disease for which rapid diagnosis and treatment are a medical emergency.[1] Despite advances in antibiotics and vaccines against many of the causative species, bacterial meningitis is still a serious concern, particularly for infants and in the developing world. Thus, as in many areas of infectious disease, there are many unmet needs for studying bacterial meningitis and the *Neisseria*, *Haemophilus*, and *Streptococcus* bacteria that cause it. These include better diagnostic methods, novel antibiotic targets, and increased understanding of how pathogens colonize hosts, invade tissues and evade the immune system.

Immunoglobulin A (IgA) is the principal immunoglobulin found in secretions that bathe mucosal surfaces. IgA thus forms the first line of antibody defence against pathogenic bacteria.[2] Several gram-positive and gram-negative bacteria including *N. gonorrhoeae*, *N. meningitidis*, *H. influenzae*, *S. sanguinis* and *S. pneumoniae* all secrete proteases that cleave the primary isotype of human IgA, IgA1 (Figure 1A).[2-3] Three independent lines of IgA1 proteases have been found in human pathogens, a striking example of convergent evolution that implies an important role in host-pathogen interactions.[4] While enzyme levels vary among isolates, high production levels of IgA1 proteases correlate with increased pathogenicity across multiple species of bacteria.[5] Cleavage of IgA1 dissociates its antigen-binding Fab domain from the Fc domain, and this prevents Fc-mediated defences such as agglutination, binding of bacteria by phagocytotic cells, and inhibition of bacterial adhesion to mucosal cells. Thus IgA1 cleavage contributes to immune evasion by these infectious bacteria.[6] Additional mechanisms by which IgA1 proteases may contribute to bacterial infection have been proposed. IgA1 proteases from *N. gonhorroeae* stimulate the production of pro-inflammatory cytokines and disrupt TNF-α signalling, suggesting roles for the protease in post-invasion virulence.[7] Cleavage of lysosomal-associated membrane protein 1 (LAMP-1) by Neisserial proteases also contributes to intracellular survival once gonococci are taken up by endosomes, and promotes trafficking across epithelial layers.[8] Taken together, all of these observations imply key roles for IgA1 proteases in the colonization, persistence and invasion of diverse bacterial pathogens, including those responsible for most cases of bacterial meningitis.

Despite this evidence, further investigations as to whether IgA1 proteases constitute true virulence factors have progressed very slowly because of the extreme selectivity of these enzymes. IgA1 proteases differ in sequence and catalytic mechanism, but all cleave IgA1 directly after various proline residues within a short "hinge" region that connects the Fab and Fc domains (Figure

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1B).[5a, 9] This hinge region is unique to IgA1 of humans and great apes, precluding the use of convenient animal antibodies as substrates. Further, while some efforts have described alternate substrates for IgA1 proteases in the cellular environment or engineered protein substrates containing the IgA1 hinge region,[10] the only robust *in vitro* substrate that has been described is human IgA1 isolated from human myeloma cells or human secretions such as breast milk. Recent studies describing cleavage of purified LAMP-1, TNF receptor II, and other host proteins have implied that IgA1 proteases may not be as selective as previously thought, and may play a wider role in bacterial invasion and virulence.[8a-c, 11] However, studies into these intriguing host-pathogen interactions have been difficult because there are no synthetic probes capable of rapid, quantitative measurement of IgA1 protease activity. This has also prevented the application of modern screening methods to the problem of finding selective IgA1 protease inhibitors.[12]

Herein we describe the design, characterization, and application of peptide substrates for diverse IgA1 proteases derived from *Neisseria*, *Haemophilus* and *Streptococcus* strains. We also demonstrate the utility of a peptide-based probe for detecting and quantifying IgA1 protease activity, and evaluate the probe as a basis for developing diagnostics and high-throughput screens for inhibitors of IgA1 proteases.

Results and Discussion

Design and evaluation of substrates based on the hinge region and autoproteolytic sites

Prior work in this field had established that short peptides corresponding to the IgA1 hinge region are not general substrates for IgA1 proteases, and that the narrow selectivity of these proteases arises from interactions with IgA1 distant from the hinge region.[3b, 5a, 10c, 13] The serine-type IgA1 proteases of gram-negative bacteria are autotransporters that cleave themselves after secretion through the bacterial outer membrane.[14] We decided to investigate whether internal cleavage sites could represent alternative starting points for designing substrates. Some cleavage by *N. gonorrhoeae* type 2 IgA1 protease ($IgAP_{Ngon2}$) of two decapeptides corresponding to $IgAP_{Ngon2}$ autoproteolytic sites have been reported, but these were not developed further.[10b] We designed candidate substrates based on internal cleavage sites from $IgAP_{Ngon2}$ and *H. influenzae* type 2 IgA1 protease ($IgAP_{Hinf2}$), as well as a 25-amino-acid peptide derived from the human IgA1 hinge region (Figure 1C).[15] To broadly test substrates among diverse, clinically relevant IgA1 proteases, we prepared five different IgA1 proteases by isolating them directly from clinically isolated strains of *N. gonorrhoeae*, *N. meningitidis*, *H. influenzae*, and *S. sanguinis*. Protease preparations were normalized for IgA1 protease activity, assigning 1x concentration to the concentration of protease that cleaved 50% of human IgA1 (30 μg/mL) after 120 min at 37°C (Figure S1). Proteases at 4x concentration were then incubated with 13.5 nanomoles of each of the four peptides **hinge**, **HI1**, **NG1** and **NG2** (Figure 1C). The fraction of each peptide remaining after 1, 8, or 24 hours was quantitated by analytical HPLC (Figure S2, Figure 2A-C).

The **hinge** peptide, which is longer than previous IgA1-derived peptides that were tested as substrates, was cleaved completely by IgA_{Hinf2} after 24 hours, only 16% by $IgAP_{Nmen2}$ after 24 hours, and not at all after 24 hours by the other three IgA proteases (Figure S3). As suggested by previous work,[10b] a peptide derived from the $IgAP_{Ngon2}$ autoproteolytic site, **NG2**, was cleaved by $IgAP_{Ngon2}$. Unexpectedly, peptide **NG2** was also cleaved by $IgAP_{Hinf2}$, $IgAP_{Nmen2}$, and $IgAP_{Ssan1}$ (Figure 2A). This result was surprising since, other than being proline-rich, peptide **NG2** does not resemble internal sites of these additional proteases. Peptide **NG1**, derived from a different $IgAP_{Ngon2}$ autoproteolytic site, was only partially cleaved by $IgAP_{Ngon2}$ after 24 hours, but was cleaved more readily by $IgAP_{Hinf2}$. Peptide **HI1** was only cleaved by the protease from which it was derived, $IgAP_{Hinf2}$, and relatively slowly compared to cleavage of **NG2** by the same protease. Thus, instead of each autoproteolytic site acting as a substrate for its respective protease, we found that one peptide, **NG2**, acts as a more general IgA1 protease substrate. We also found that, despite being normalized with respect to activity towards human IgA1, different IgA1 proteases can cleave proline-rich peptides to different extents, with $IgAP_{Hinf2}$ being the most active towards short peptides. The different proteases also show distinct substrate selectivities among proline-rich peptides.

As noted above, cleavage of immunoglobulins by IgA1 proteases is notoriously selective. The fact that IgA1 proteases cleave selectively among peptides **hinge**, **NG1**, **NG2**, and **HI1** implied that peptide cleavage was recapitulating some of the features of IgA1 cleavage. We used MALDI-TOF mass spectrometry to verify this selectivity and to identify the cleavage sites (Figure S4). Specific cleavage of **NG2** between Pro8 and Ala9 was observed upon incubation with $IgAP_{Ngon2}$, $IgAP_{Nmen2}$, $IgAP_{Hinf2}$, and $IgAP_{Ssan1}$. Similar specific cleavage was observed between Pro7 and Ser8 of **NG1** when incubated with $IgAP_{Hinf2}$. After 8 hours of incubation with $IgAP_{Hinf2}$, small amounts of peptide fragments were observed indicating a secondary cleavage site between Pro5 and Arg6 of **NG2**, but otherwise protease reactions were highly selective for specific peptides and specific cleavage sites. These sites correspond to the native autoproteolytic sites, further supporting that the observed cleavage events represent the intrinsic selectivities of these IgA1 proteases.

These data are the first demonstration of a synthetically prepared substrate for IgA1 proteases from *N. meningitidis*, *H. influenzae*, and *S. sanguinis*. Also, we found that disparate IgA1 proteases have surprising common substrate preferences. This was unexpected because the proteases have different native cleavage sites on human IgA1 (Figure 1B), and because they range from serine proteases with high sequence homology ($IgAP_{Nmen2}$ and $IgAP_{Ngon2}$) to an independently evolved metalloprotease with no homology to the serine proteases ($IgAP_{Ssan1}$). These observations represent an opportunity to use these peptides as general sensors for IgA1 protease activity. In fact, trace amounts of **NG2** cleavage by $IgAP_{NMen1}$ can be observed in HPLC chromatograms (see Figure S5), though not enough to quantitate the loss of **NG2** through peak integration. This gave further evidence that peptide **NG2** may be a more universal substrate for IgA1 protease activity, and led us to pursue it further as a diagnostic tool.

Design and evaluation of a turn-on fluorescent sensor for IgA1 protease activity

We next sought to increase assay sensitivity by developing a fluorescent probe based on **NG2**. A series of truncated versions (peptides **NG2.1**-**NG2.5**, Figure 1C) were tested in the HPLC-based assay with $IgAP_{Hinf2}$ to determine the minimal sequence that acted as an IgA1 protease substrate (Figure 2D). Peptide

NG2.2 was selected as a shorter substrate that was still efficiently cleaved. Next, probe **F2.2** was designed based on **NG2.2**, incorporating an EDANS fluorescence donor at the C-terminus and a Dabcyl quencher at the N-terminus. To test the probe's suitability as a sensor for protease activity, we first incubated it with the aggressive protease Proteinase K (ProtK). ProtK incubation of only 5 minutes produced an EDANS fluorescence that was 5-fold greater than probe alone (Figure 3A), and this value did not increase after longer incubation. Thus, we took this as an upper bound for the assay. We then incubated **F2.2** with five different IgA1 proteases for 120 min at 0.25x concentration, or 1/16th the concentration used in HPLC-based assays (Figure 3A, light gray bars). The largest signal was observed for $IgAP_{Hinf2}$, as expected from HPLC-based assays, and its intensity was 34% of the maximum signal observed using ProtK. Smaller signals were observed when **F2.2** was incubated with $IgAP_{Ngon2}$, $IgAP_{Nmen1}$, $IgAP_{NMen2}$, and $IgAP_{Ssan1}$. When 4x concentrations of IgA1 proteases were used, all four of these proteases produced more robust signals ranging from 20%-60% of maximum (Figure 3A, dark gray bars). This included $IgAP_{NMen1}$, whose cleavage of full-length **NG2** was barely detectable in the HPLC-based assay (Figure 2A).

The linearity of the probe's response was tested by incubating 4x $IgAP_{NMen2}$ with different concentrations of probe (0.375 to 3 μM **F2.2**) and for different incubation times (2 min to 8 h). Probe response was linear with time and probe concentration, and the length of time required for signal saturation increased with probe concentration (Figure 3B). Similar results were obtained with $IgAP_{Hinf2}$ (Figure S6). Thus, we concluded that **F2.2** was a robust *in vitro* probe for detecting the activity of diverse IgA1 proteases.

Interference from background proteases are a concern for potential applications of peptide substrates using complex biological samples, such as rapid diagnosis of bacterial meningitis. Thus, we evaluated whether the fluorescence assay would be selective enough for detecting IgA1 proteases in human biological fluids. Fluorescence assays were repeated using IgA1 proteases at 0.25x and 4x concentrations added to human serum (Figure 3C). High intrinsic fluorescence of the serum proved difficult to subtract out, leading to poor overall assay performance. Also, some **F2.2** cleavage (roughly 21%) was observed after 120 min in unaltered human serum (Figure S7). Since the current "gold standard" for diagnosing bacterial meningitis involves culturing CSF,[1c, 16] we also tested the robustness of the probe in detecting IgA1 proteases added to human CSF. CSF had much less intrinsic fluorescence, and very little cleavage of the probe was observed after 120 min at 37 °C (Figure S7). We performed assays with IgA1 proteases at 0.25x and 4x concentrations in CSF, and observed signal intensities and signal-to-noise ratios that were very similar to those observed in buffer (Figure 3D compared to Figure 3A). We concluded that **F2.2** can detect IgA1 protease activity in CSF, and has potential as a diagnostic tool for detecting the presence of diverse pathogens.

The presence of IgA1 protease in serum and CSF is highly likely in cases of acute meningitis. Pathogenic strains of many bacteria secrete large amounts of IgA1 proteases, and antibodies recovered from acute and post-infection patients have IgA1 protease-neutralizing activity.[17] However, the clinically relevant concentration range for IgA1 proteases in CSF during a meningeal infection is currently unknown. This lack of knowledge is the direct result of the inadequate methods available for quantitating IgA1 protease activity. To begin applying our probes to these and other important questions, it was necessary to quantitatively benchmark their performance using recombinant, active forms of IgA1 proteases. We expressed and purified a recombinant form of *H. influenzae* type 1 IgA1 protease (r-$IgAP_{Hinf1}$) as we previously reported.[5a, 18] This serine protease has some homology to the *H. influenzae* type 2 protease $IgAP_{Hinf2}$, as well as with $IgAP_{NMen1}$ and $IgAP_{NMen2}$, but $IgAP_{Hinf1}$ cleaves at a different IgA1 site from these other proteases (Figure 1B).[9b, 19] When r-$IgAP_{Hinf1}$ was normalized with respect to IgA1 cleavage, we observed that 0.72 μg/mL (6.6 nM) of recombinant protease was required to cleave 50% of human IgA1 (30 μg/mL) after 2 hours at 37°C. When **NG2** was incubated with 2.9 μg/mL r-$IgAP_{Hinf1}$, the same relative activity level used for other IgA1 proteases in HPLC-based assays, only trace cleavage was observed by HPLC (as observed for $IgAP_{Nmen1}$, Figure S5). Increasing the amount of purified $IgAP_{Hinf1}$ to 21.6 μg/mL resulted in 80% cleavage of **NG2** after 24 hours at 37°C, with the same site selectivity as detected by mass spectrometry. Next, we incubated serial dilutions of r-$IgAP_{Hinf1}$ with **F2.2** for 120 min at 37°C, and observed a dose-dependent signal (Figure 4A). Based on these results, we conclude that the limit of detection of this assay is roughly 0.7 μg/mL r-$IGAP_{Hinf1}$. 2.9 μg/mL protease could also be detected when r-$IgAP_{Hinf1}$ was added to human CSF (Figure S7). These results demonstrate that **F2.2** will be a useful, quantitative tool for quantifying IgA1 protease activity in purified preparations and in human CSF samples.

Conclusion

In this report, we describe peptide probes for the rapid, sensitive and quantitative detection of IgA1 protease activity. An important application of the present work will be the development of new tools for diagnosis of bacterial meningitis.[20] Peptide-based assays may have advantages over other methods for diagnosing meningeal infections. Culturing is slow, and microscopic examination of stained CSF is limited by the ability to find and characterize the infecting bacteria. PCR and ELISA-based techniques have not supplanted culturing as a primary means of diagnosis.[1c] Current culturing methods require 1 mL or more of CSF and take up to 2 days, but the volume of CSF used for the fluorescence assay was only 25 μL and required 120 min or less.[16a] Also, while the persistence of IgA1 proteases has not been quantified, detection of IgA1 proteases may provide a unique means of diagnosis even after administration of antibiotics. Future work will use clinical samples to validate whether probe **F2.2** can be used to positively identify CSF samples from patients with bacterial meningitis. Additional probes will also be developed in order to increase sensitivity, to decrease cleavage by unrelated proteases such as those found in serum, and also to more accurately distinguish among different IgA1 proteases. These studies will be helped by the structure-activity relationships begun here.

The substrates and fluorescent probe we describe are also long-needed tools for understanding the roles of IgA1 protease in bacterial virulence and host-pathogen interactions. Future applications could use these assays to measure IgA1 protease levels in human serum, CSF, sputum, and even saliva in different states of health and disease. We can also explore recently proposed roles of IgA1 proteases in intracellular trafficking and survival,[8] and potential involvement of IgA1 proteases in the bronchial colonization of patients with chronic obstructive pulmonary disorder.[21]

Another open question regarding IgA1 proteases is whether they may be viable pharmacological targets for controlling bacterial infections.[6a, 12] Because these are the first synthetic probes identified for IgA1 proteases, the fluorescence assay described herein represents the first assay suitable for high-throughput screening for IgA1 protease inhibitors. To verify the utility of the assay for high-throughput screens, we performed the assay in a 384-well format using $IgAP_{Hinf2}$ and observed good signal-to-noise and reproducibility, with a Z-factor of 0.70 (Figure 4B). Continued progress towards selective IgA1 protease inhibitors will allow definitive assessments of the roles of IgA1 proteases in infection and virulence, and will explore the possibility of targeting them to diagnose or treat devastating meningeal infections.

Experimental Section

Peptide synthesis. All peptides were synthesized using standard Fmoc solid phase peptide synthesis using solvents and reagents from Anaspec and EMD-Millipore. Fmoc-tyrosine(tBu)-loaded Wang resin, or EDANS Novatag™-resin for **F2.2**, was extended by cycles of deprotection using 20% piperidine and coupling using of diisopropylethylamine (13 equivalents), *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU, 4.8 equivalents) and 1-hydroxy-7-azabenzotriazole (HOAt, 5 equivalents), or O-benzotriazole-N,N,N',N'-tetramethyluronium-hexafluorophosphate (HBTU, 4.8 equivalents) and 1-hydroxybenzotriazole (HOBt, 5 equivalents). Residues following proline were double-coupled to promote coupling efficiency. Dabcyl-succinimidyl ester (EMD-Millipore, 2 equivalents) were coupled to the N-terminus of **F2.2** in the presence of HOBt (1.25 equivalents) for 12 hours. Following cleavage using a standard trifluoroacetic-acid-based cocktail, peptides were purified to >95% purity by reverse-phase HPLC using a preparatory-scale C8 column and a water-acetonitrile gradient in 0.1% trifluoroacetic acid. Peptide purity and identity were confirmed using analytical HPLC and MALDI-TOF mass spectrometry.

P*reparation of IgA1 proteases from clinically isolated bacterial strains.* *H. influenzae* strain E, (ATCC #8142, producing Type 2 IgA1 protease) was grown overnight in Brain Heart Infusion (BHI) broth (BD, Franklin Lakes, NJ, USA) supplemented with hemin (10 μg/ml) and nicotinamide adenine dinucleotide (NAD). *N. gonorrhoeae* strains MKB and MS11 (both producing type 2 IgA1 proteases), and *N. meningitidis*, strains 1C (producing type 1 IgA1 protease) and 2R (producing type 2 IgA1 protease) were all clinically isolated strains previously reported.[22] Each of these Neisserial strains was grown overnight in BHI enriched with 1% IsoVitalex (BD, Franklin Lakes, NJ, USA). *S. sanguinis* (strain ATCC #10556) was grown in Todd-Hewitt broth as described previously.[6c] IgA1 proteases were isolated by growing each bacterial strain was in a 10-liter New Brunswick Bioflo 4500 fermentor equipped with a microprocessor to control pH, airflow, and temperature. After overnight growth, culture medium containing the enzyme was separated from the bacterial mass using a Pellicon tangential flow membrane filtration system with a 0.45 micron filter. A second Pellicon unit with a 30K molecular weight cutoff membrane was used to concentrate the filtrate from 10 liters to about 500 mL and to equilibrate it in 25 mM Tris/HCl buffer, pH 7.5, with 0.025% sodium azide. These concentrated enzyme preparations were divided in aliquots, and stored in -80 °C.

Expression and purification of recombinant, type 1 IgA1 protease from H. influenzae *(r-$IgAP_{Hinf1}$).* r-$IgAP_{Hinf1}$ was expressed in *E. coli* strain BLR (DE3) using a pET vector cloned with a major fragment of the *iga* gene of *H. influenzae* strain Rd6H, described previously.[18] This fragment of the *iga* gene encodes only the mature IgA1 protease domain, amino acids 26-1014, and excludes the N-terminal signal peptide and the C-terminal helper beta-domain. At log phase, IPTG (0.5-1.0 mM) was added to stimulate the enzyme production. Active IgA1 protease expressed in this way accumulated in inclusion bodies. Cells were harvested, and the soluble IgA1 protease was obtained after lysis with BugBuster Master Mix (EMD Millipore, USA). IgA1 protease in the supernatant was purified on a Ni-NTA column according to manufacturer's protocols (Qiagen).[23]

HPLC-based peptide cleavage assays. Peptide (13.5 nmol) from concentrated DMSO stock was incubated with four times the normalized IgA1 protease concentration (4x) in 25 mM Tris, pH 7.5 and 150 mM NaCl at 37°C for incubation times of 24 h, 8 h or 1 h. Assays were stopped with four reaction volumes of dimethyl sulfoxide (DMSO), conditions which eliminated protease activity. Samples were analyzed by RP-HPLC on a C18 column using a Varian ProStar HPLC with automated sample injection. Identical controls that lacked IgA1 protease were injected before and after each automated run of 8-12 analyses to validate quantified peak values. If no cleavage was observed at 24 h, assays with shorter incubation times were not performed.

Fluorescence assay setup. Fluorescence assays were performed in flat-bottom, black 384-well plates with 40 μL assay volume in each well. **F2.2** (0.75 μM) was incubated with one-fourth or four times the normalized concentration (0.25x or 4x) IgA1 protease in Tris-NaCl buffer. The DMSO concentration in all the assays was maintained below 1% *v/v*. The EDANS fluorescence emission at 485 nm was measured following excitation at 340 nm at various time points. For assays in serum and CSF, 5 μl of human serum (Sigma-Aldrich) or human CSF (de-identified samples provided by Clinical Microbiology Laboratory, Department of Pathology, Tufts Medical Center) was used in each assay along with Tris-NaCl buffer, maintaining the total volume of the assay at 40 μL. Controls included buffer/serum/CSF alone, probe alone, IgA1 protease alone, and probe and buffer/serum/CSF without protease. **F2.2** incubated with Proteinase K was used as a positive control for all assays. To establish reproducibility and calculate a Z-factor, the 40 μL-volume assay was repeated with 96 wells of **F2.2** in Tris/NaCl buffer and 88 wells with 4x $IgAP_{Hinf2}$. EDANS emission at 485 nm following excitation at 340 nm was measured after 120 minutes at 37°C.

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Figure 1. Cleavage of IgA1 and designed peptide substrates. A) Diagram of the domain structure of dimeric human IgA1. Cleavage of IgA1 at the hinge regions disconnects the Fab from the Fc domain, subverting the agglutination and clearance functions of the antibody. B) Sequence of the IgA1 hinge region, with established cleavage sites for several IgA1 proteases indicated in boldface. C) Sequences of peptides evaluated as IgA1 protease substrates and fluorescence probes. Peptide bonds following bolded prolines are IgA1 protease cleavage sites confirmed by mass spectrometry.

Figure 2. Peptide cleavage assays identify substrates for several IgA1 proteases. A) Percentage of peptide **NG2** remaining after incubation with the indicated IgA1 proteases for 1 hour (white), 8 hours (light gray), and 24 hours (dark gray). Asterisks indicate zero values. B) Percentage of peptide **NG1** remaining in identical assays. C) Percentage of peptide **HI1** remaining in identical assays. Asterisk indicates a zero value. D) Cleavage of truncated analogs of **NG2** by $IgAP_{Hinf2}$. Sequences of peptides **NG2.1**-**NG2.5** are given in Figure 1c. Error bars represent standard deviation from three independent trials.

Figure 3. Fluorescence assays characterize a probe for IgA1 protease activity. A) Detection of IgA1 protease activity in buffer using 0.75 μM **F2.2**. B) Linear response of the probe is demonstrated by incubating **F2.2** at the indicated concentration, for the indicated time, with 4x $IgAP_{NMen2}$. C) Detection of IgA1 protease activity using 0.75 μM **F2.2** in human serum, to which we added the indicated concentrations of different IgA1 proteases. D) Detection of IgA1 protease activity using 0.75 μM **F2.2** in human cerebrospinal fluid, to which we added the indicated concentrations of different IgA1 proteases. Error bars represent standard deviations from three independent trials.

Figure 4. Applications of probe F2.2. A) Serial dilutions of recombinant $IgAP_{Hinf1}$ incubated with 0.75 μM **F2.2** allow quantitative benchmarking of probe sensitivity. Error bars are standard deviations of three independent trials. B) Scatter plot demonstrating probe robustness in a 384-well format suitable for high-throughput screening. Data points for probe only are shown as circles, and data points for probe plus 4x $IgAP_{NMen2}$ are shown as squares.

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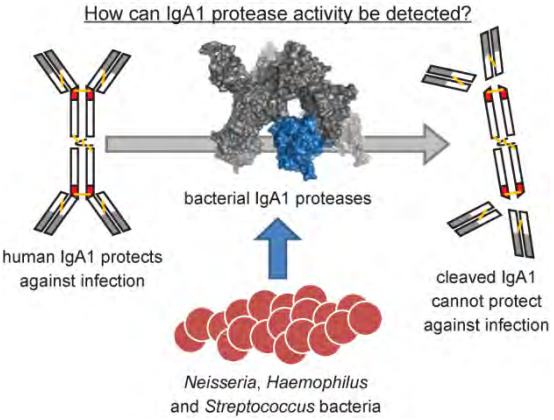
Diverse bacterial pathogens secrete immunoglobulin A1 (IgA1) proteases that assist in colonization, invasion, and immune evasion (see figure). Here we describe the first synthetic substrates and sensors for IgA1 proteases from diverse bacterial strains. These are applied to measuring IgA1 protease activity in buffer and in human cerebrospinal fluid, and in a high-throughput screen.



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Supplementary figures.

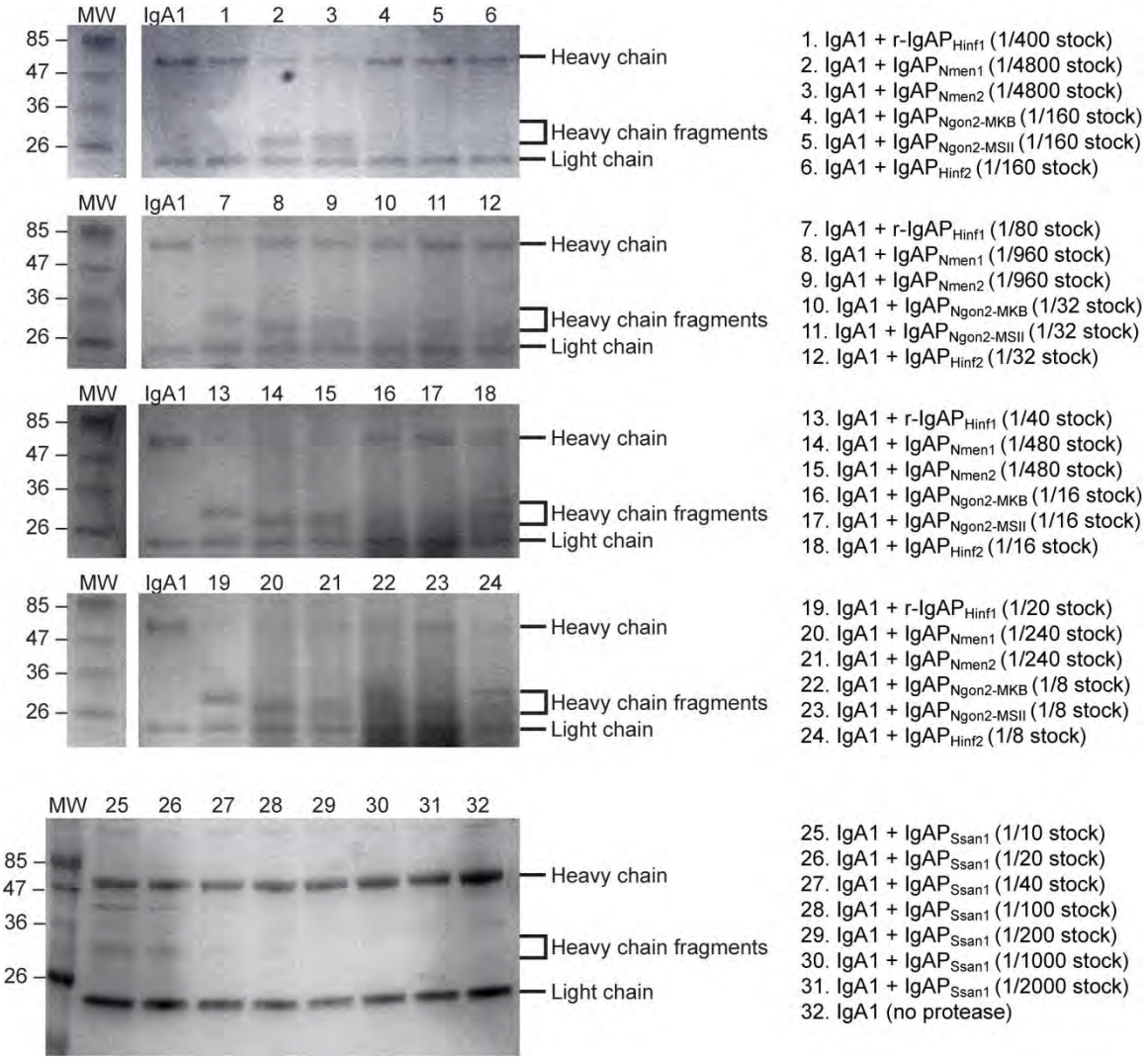


Figure S1. Sample normalizations of IgA protease preparations using human IgA1. Concentrated enzyme preparations were diluted from stock solutions by the indicated fractions and incubated with human IgA1 (30 μg/mL) for 2 hours at 37 °C in 25 mM Tris, pH 7.5 and 150 mM NaCl at 37°C. Samples were loaded onto 4-15% gradient polyacrylamide gels and Coomassie blue stain was used to visualize protein bands. The disappearance of the band corresponding to the heavy chain was quantified using Quantity One software. Local background subtraction and internal IgA1 standards, shown in the gels above in the lanes labeled IgA1, were used to ensure reproducibility. IgAP$_{Ngon2-MSII}$ was an alternative type 2 *N. gonorrhoeae* produced from strain MS11 (ATCC 49759), but was not included in further analysis.

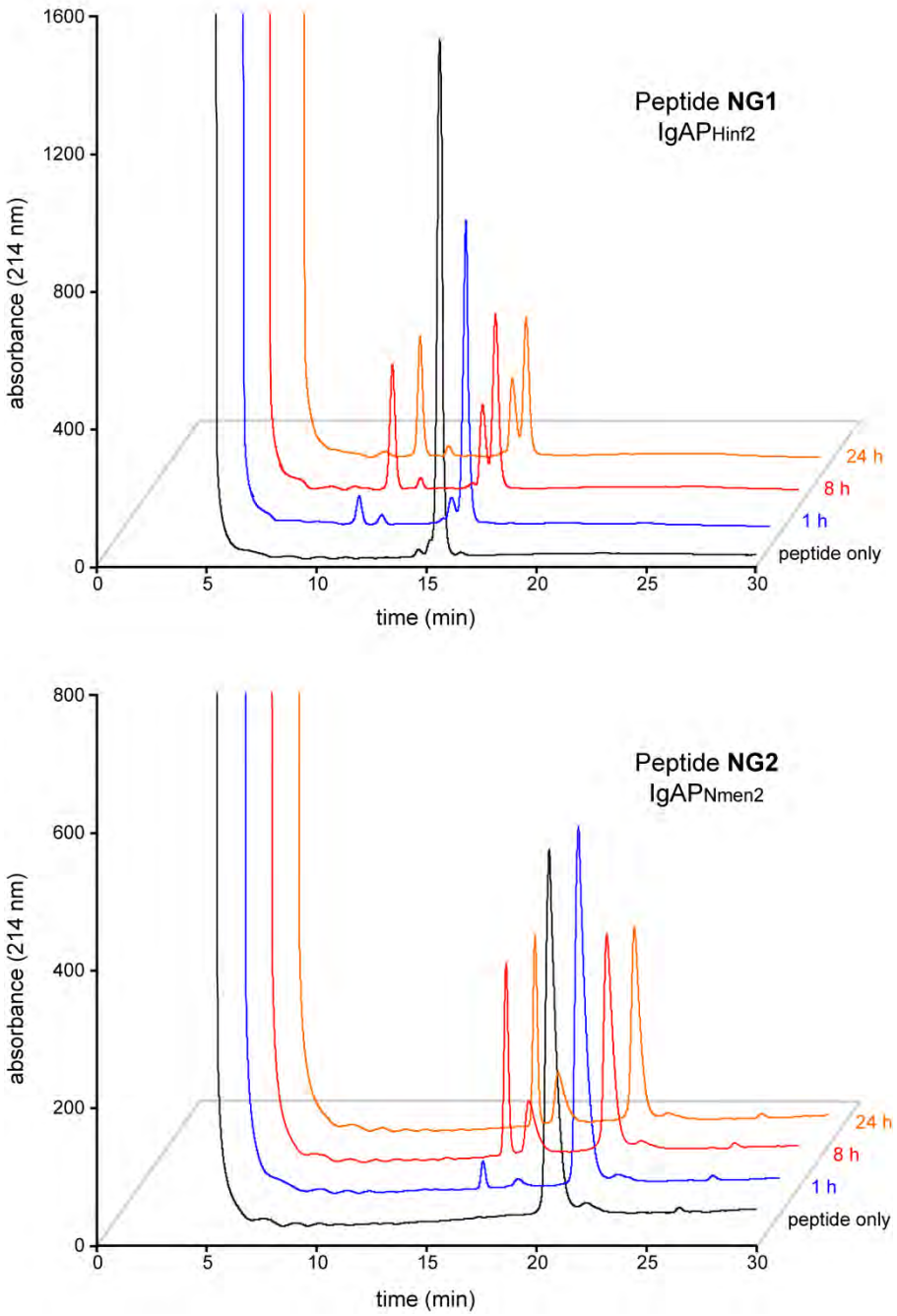


Figure S2. Sample chromatograms of HPLC cleavage assays. 13.5 nmol of peptide from concentrated DMSO stock was incubated with four times the normalized IgA1 protease concentration (4x) in 25 mM Tris, pH 7.5 and 150 mM NaCl at 37°C for incubation times of 24 h, 8 h or 1 h. Assays were stopped with four reaction volumes of dimethyl sulfoxide (DMSO), conditions which eliminated protease activity. Samples were analyzed by RP-HPLC on a C18 column using a Varian ProStar HPLC with automated sample injection. The DMSO concentration in all the assays was maintained below 2% *v/v* in the final assay, which did not affect protease activity (see Figure S6). Identical controls that lacked IgA1 protease (peptide only) were injected before and after each automated run of 8-12 analyses to validate quantified peak values and to ensure reproducibility. A minimum of three independent cleavage reactions were performed in this manner for each peptide-protease pair.

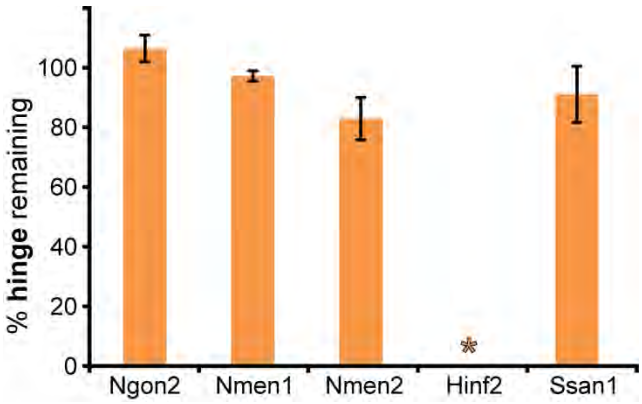
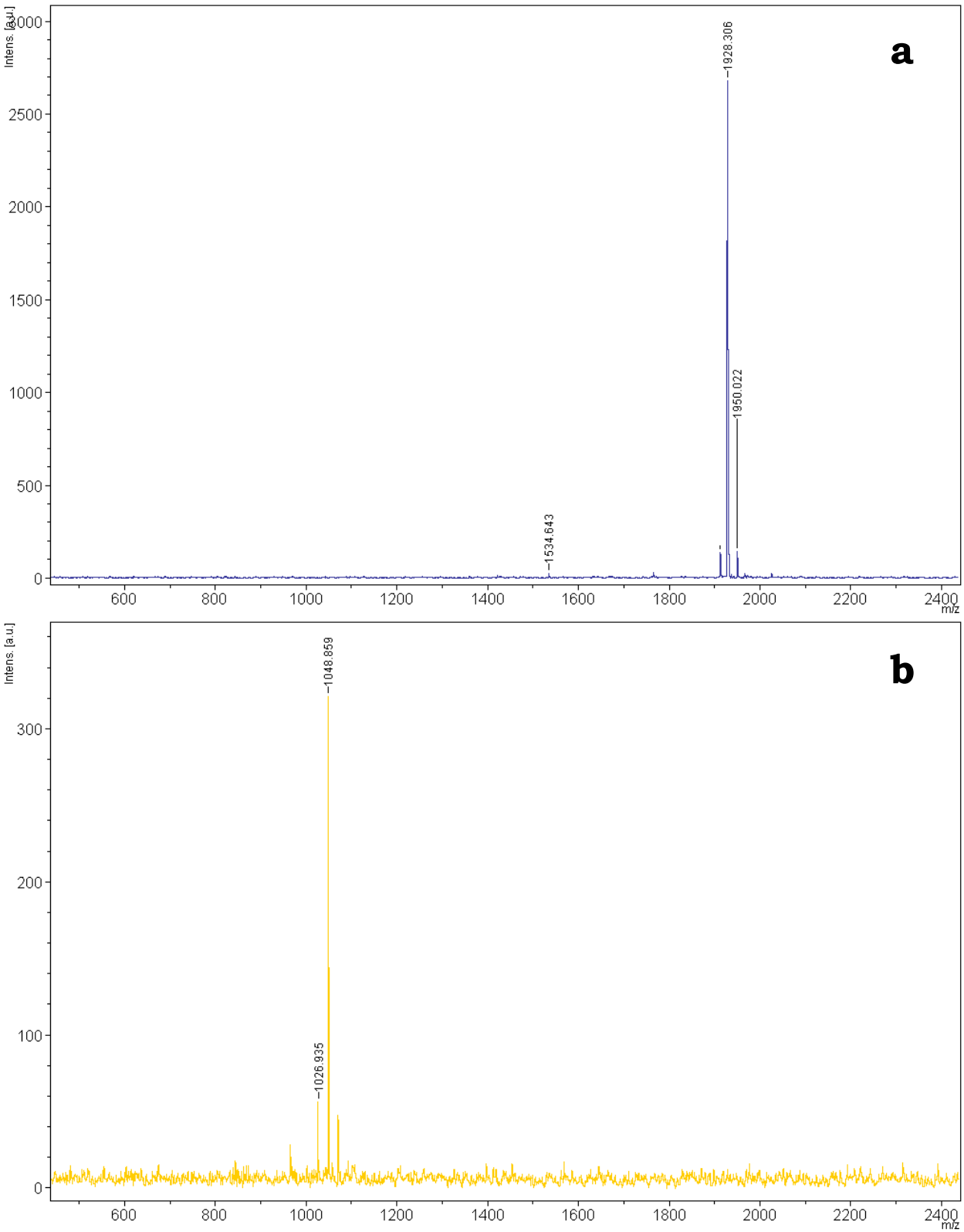


Figure S3. Cleavage of a 25-residue peptide derived from the IgA1 hinge region by IgA proteases. Peptide **hinge** (see Figure 1c) was incubated with the IgA proteases indicated for 24 hours and analyzed by HPLC as described. Error bars indicate standard deviations from three independent trials. Asterisk denotes a zero value.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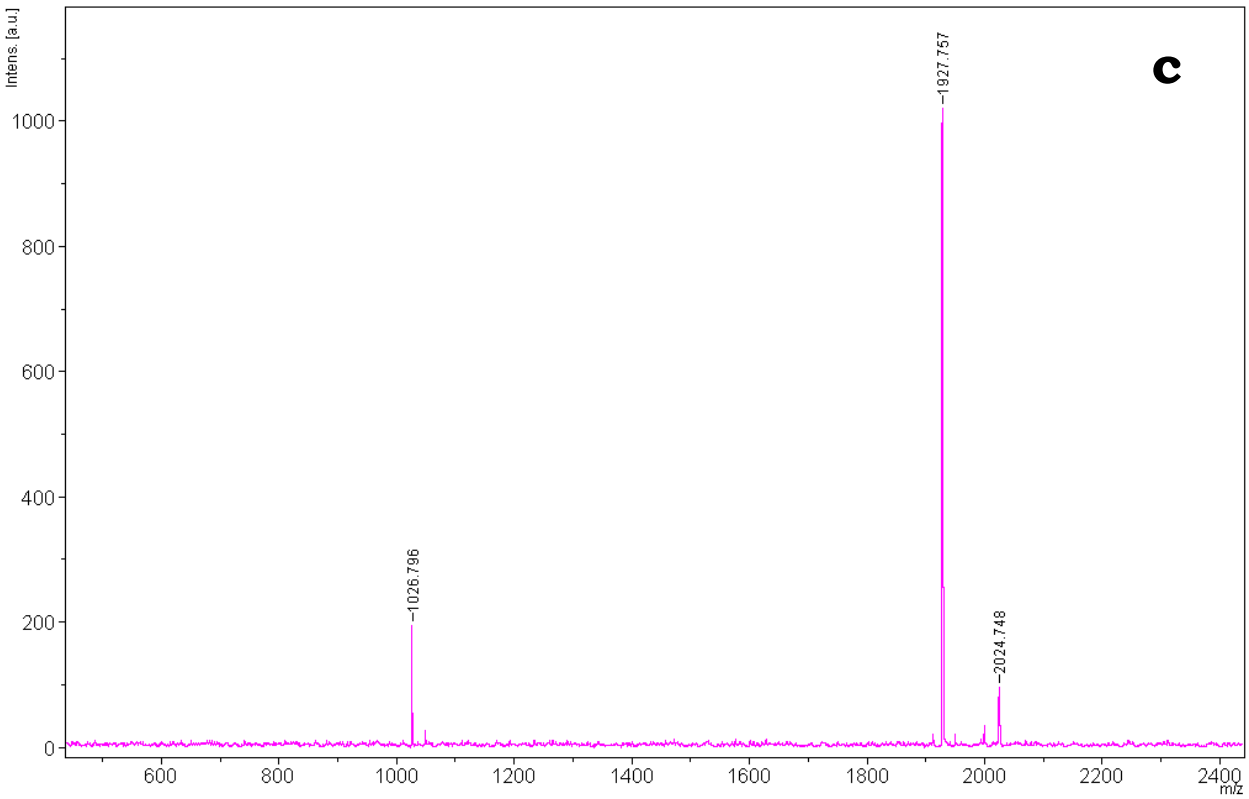
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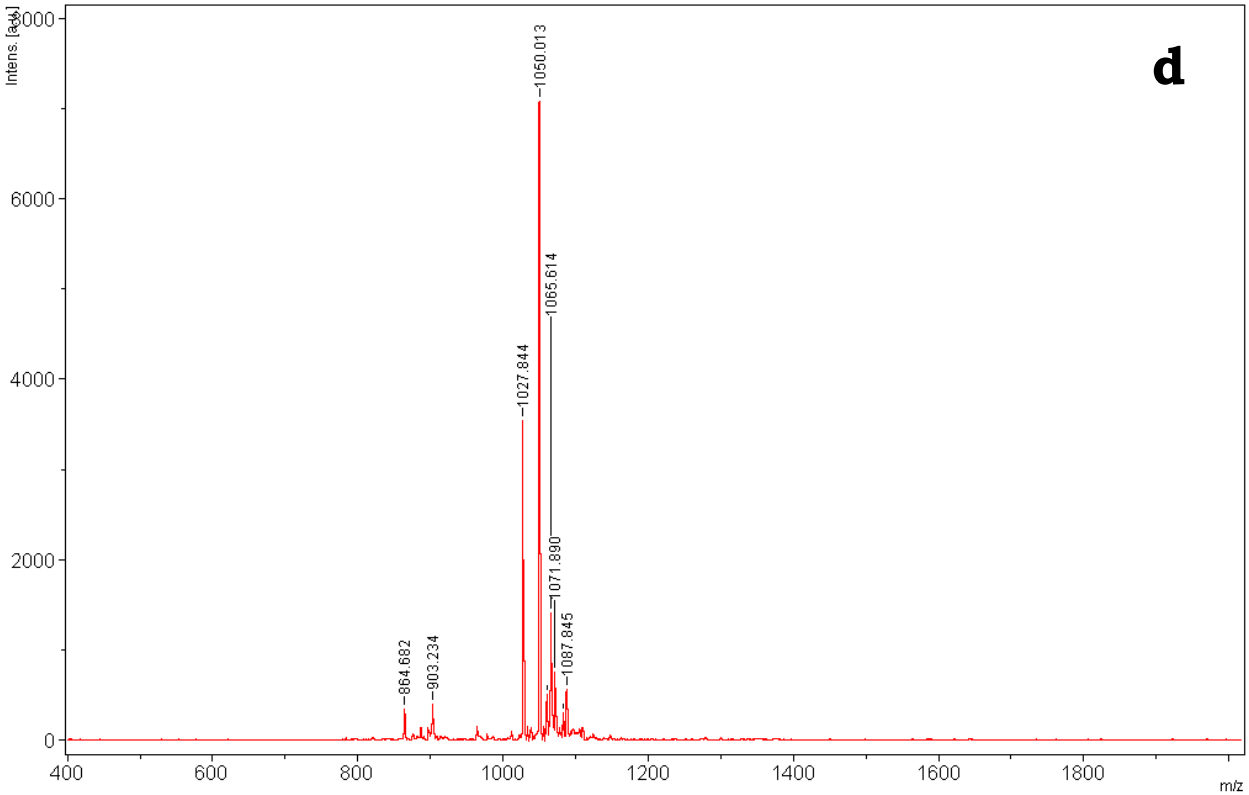
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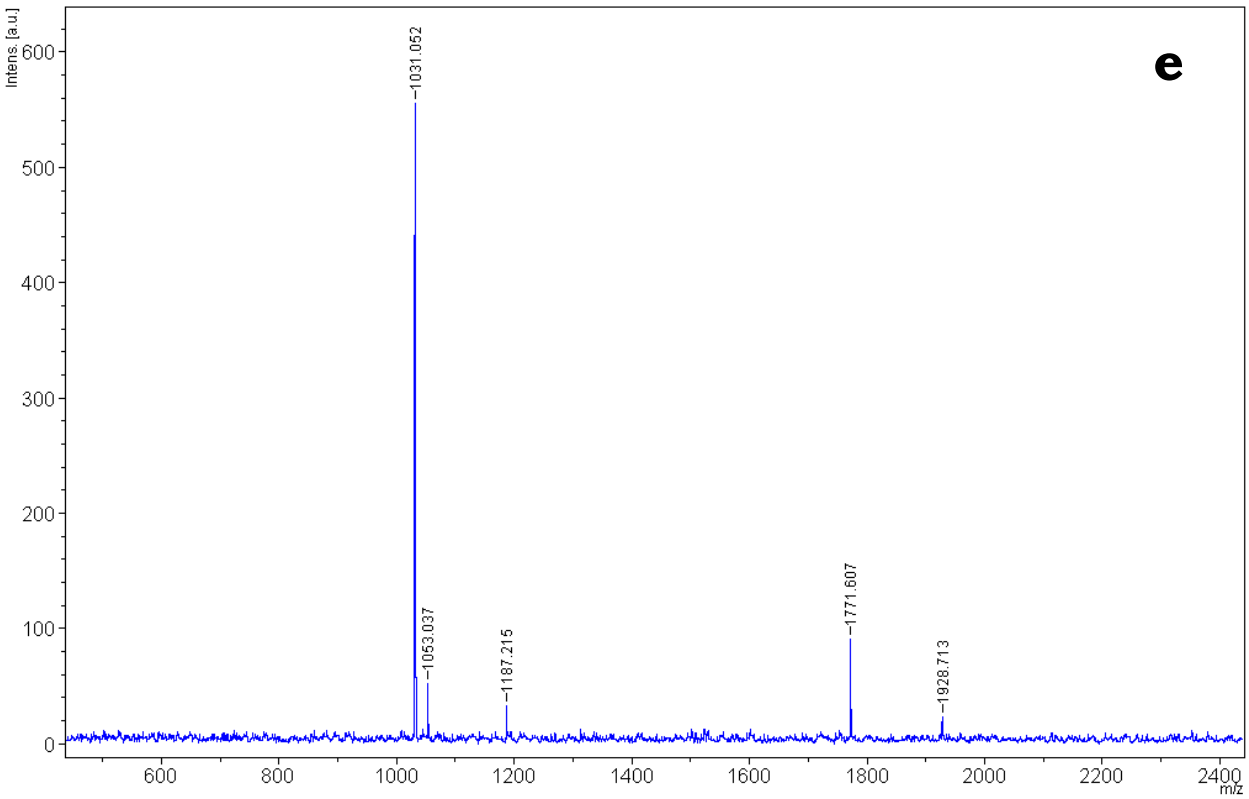
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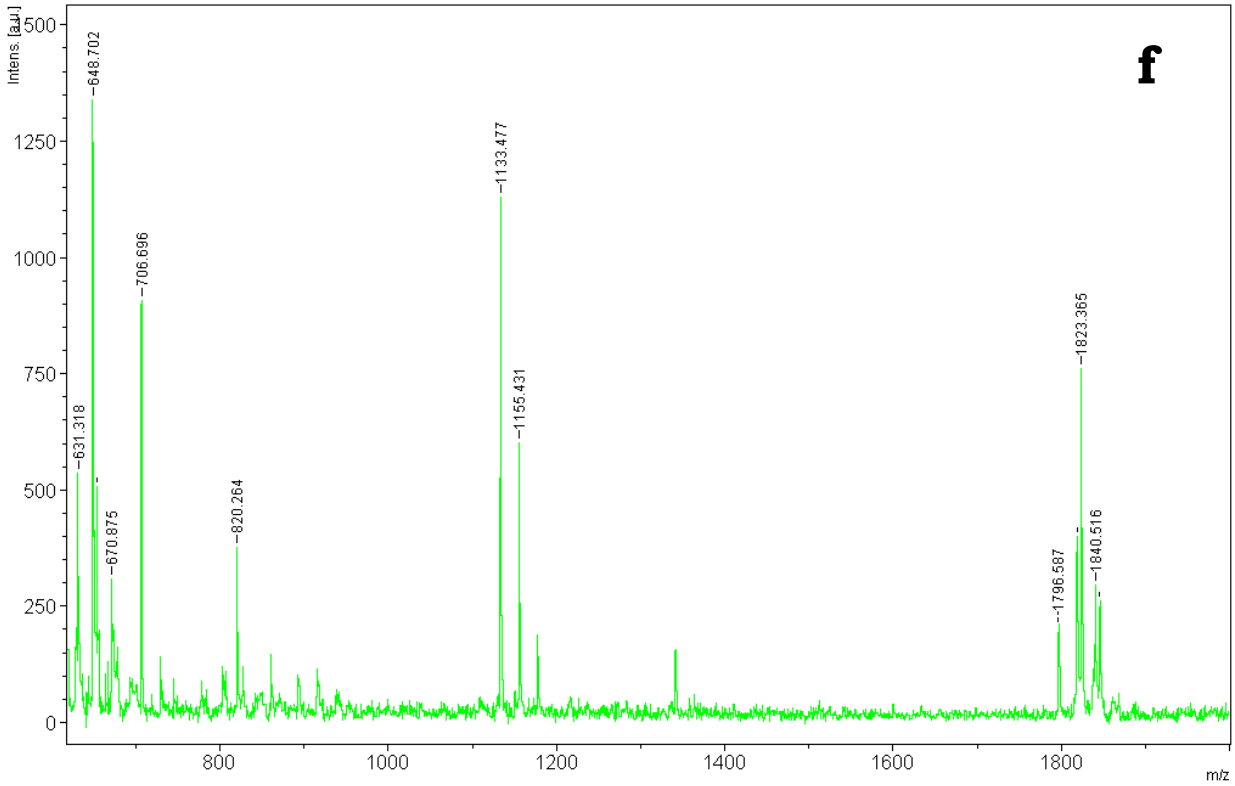
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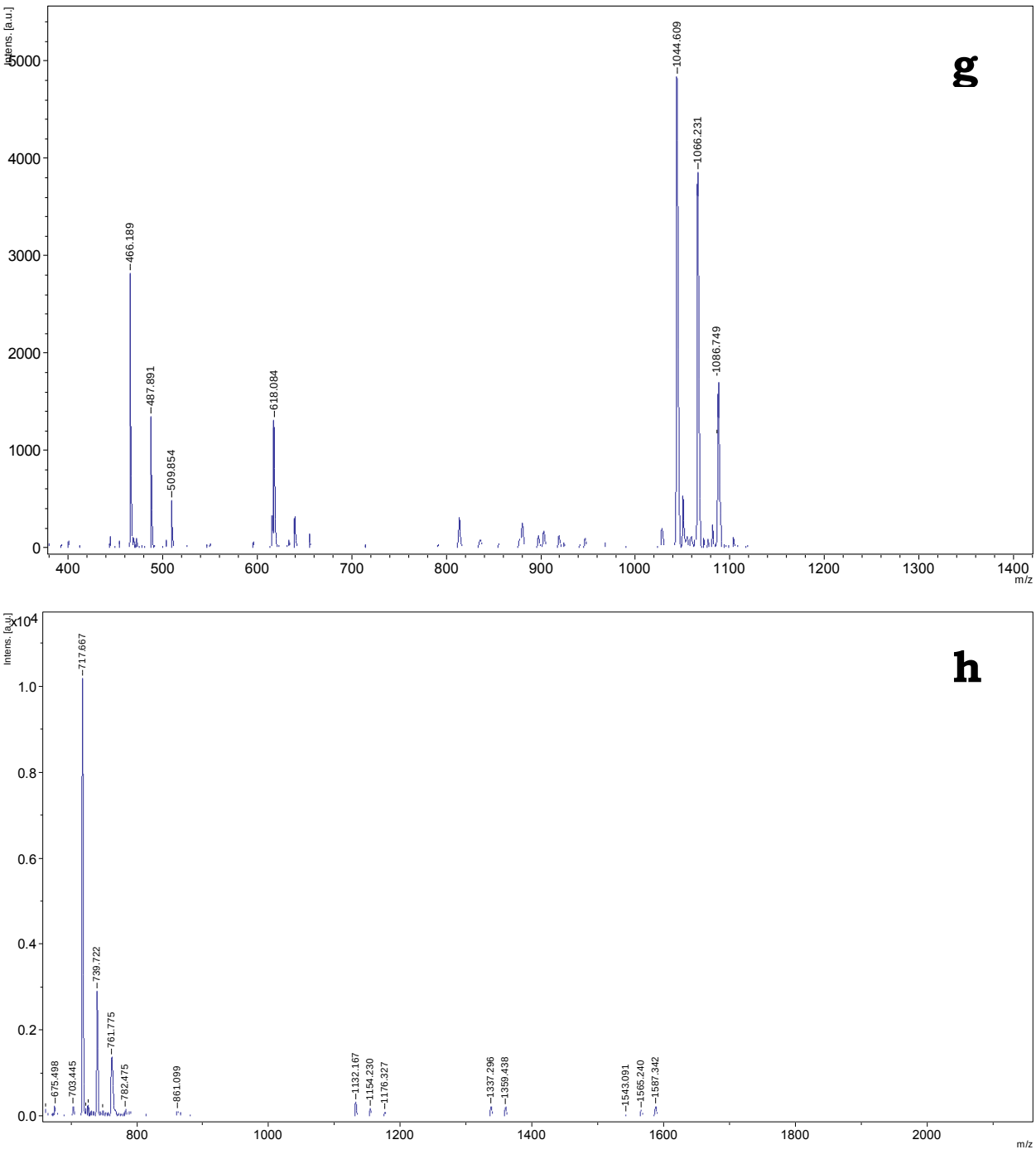

Figure S4. Identification of peptide fragments by MALDI-TOF mass spectrometry. Cleaved peptides were analyzed by MALDI-TOF to identify cleavage sites. (a-e) Fragments of **NG2** identified by MALDI from incubation with: (a) no protease, (b) $IgAP_{Ngon2}$, (c) $IgAP_{NMen2}$, (d) $IgAP_{Hinf2}$, (e) $IgAP_{Ssan1}$.

NG2 $(MW+H^+)_{calc}$ = 1927.2, fragment APVFSLDDY $(MW+H^+)_{calc}$ = 1027.1. For these data, **NG2** was incubated with or without protease for 24 h as described for HPLC assays, except for $IgAP_{Hinf2}$ which was incubated with **NG2** for 1 h. (f) Fragments of **NG1** identified by MALDI after incubation with $IgAP_{Hinf2}$ for 8 h as described for HPLC assays. **NG1** $(MW+H^+)_{calc}$ = 1827.9, fragment SPQANQAEEY $(MW+H^+)_{calc}$ = 1137.1, fragment NIVVAPP $(MW+H^+)_{calc}$ = 709.8. (g) Fragments of **NG2.2** identified by MALDI after incubation with $IgAP_{Hinf2}$ for 1 h as described for HPLC assays. **NG2.2** $(MW+H^+)_{calc}$ = 1044.2, fragment PRPP $(MW+H^+)_{calc}$ = 466.6, fragment APVFY $(MW+H^+)_{calc}$ = 596.7, $(MW+Na^+)_{calc}$ = 618.7. (h) Fragments of **F2.2** identified by MALDI after incubation with $IgAP_{NMen2}$ for 4 h as described for HPLC assays. **F2.2** $(MW+H^+)_{calc}$ = 1544.2, fragment Dabcyl-PRPP $(MW+H^+)_{calc}$ = 716.9, $(MW+Na^+)_{calc}$ = 738.9, fragment APVFY-Edans $(MW+H^+)_{calc}$ = 844.0, APVFY-Edans $(MW+Na^+)_{calc}$ = 866.0.

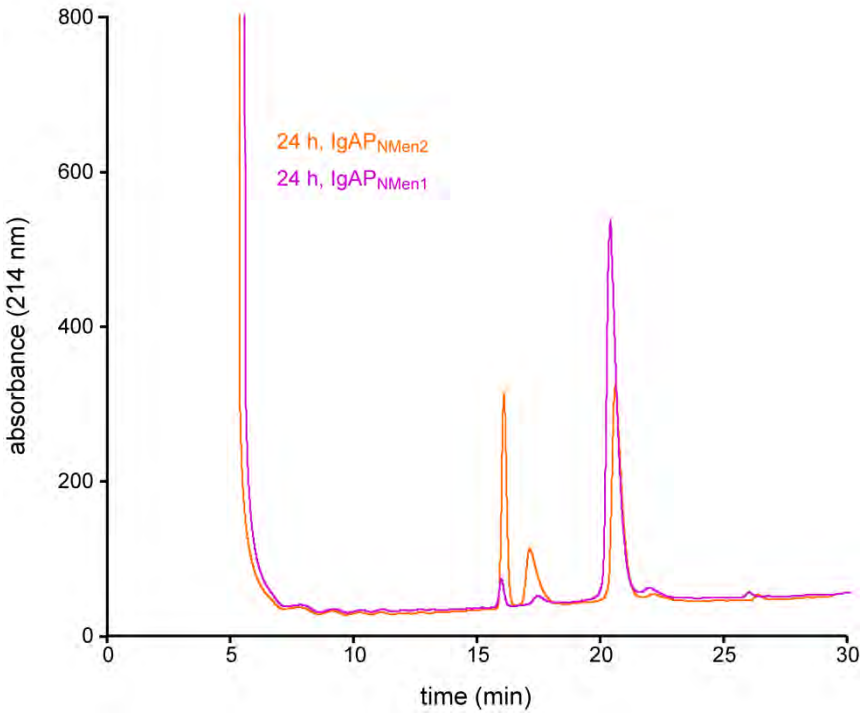

Figure S5. Evidence for trace amounts of cleavage of NG2 by $IgAP_{NMen1}$. Overlayed HPLC traces of peptide **NG2** after 24-hour incubation with $IgAP_{NMen2}$ (orange), and **NG2** after 24-hour incubation with $IgAP_{NMen1}$ (magenta). While the decrease in the **NG2** peak near 20.8 minutes was not enough to quantitate selective **NG2** cleavage by $IgAP_{NMen1}$, the appearance of the product peaks near 16 and 17 minutes match those observed after incubation of **NG2** with $IgAP_{NMen2}$. This indicates trace amounts of cleavage by this protease, which is detectable in a fluorescence assay with **F2.2** (main text, Figure 3).

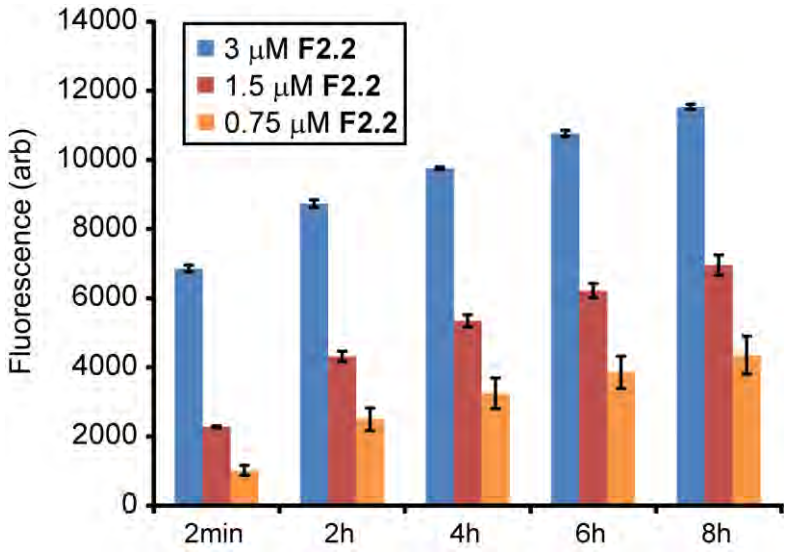

Figure S6. Similar to Figure 3b, linear response of the probe is demonstrated by incubating **F2.2** at the indicated concentration, for the indicated time, with 4x $IgAP_{Hinf2}$. Error bars show standard deviation from three independent trials.

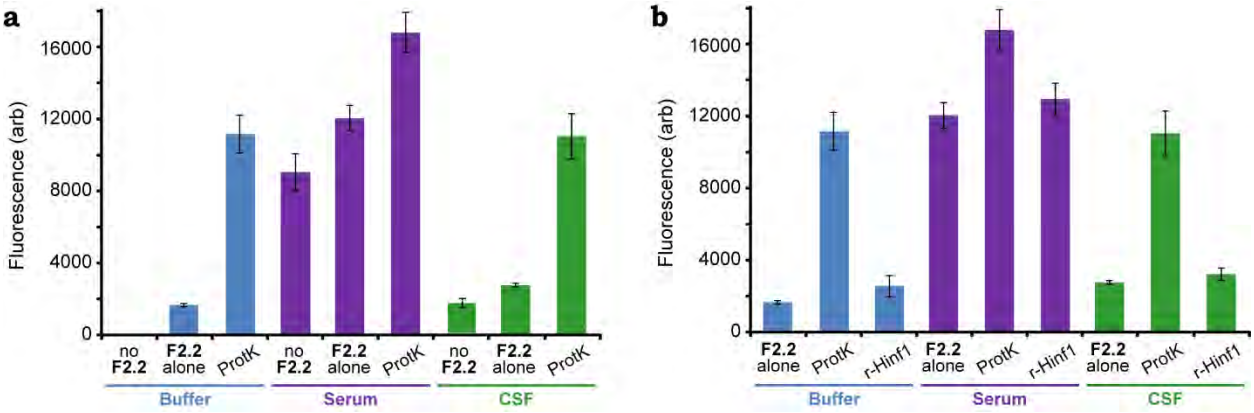

Figure S7. Performance of probe F2.2. (a) Fluorescence (excitation 340 nm, emission 485 nm) of background (buffer, serum or CSF) alone, probe **F2.2** alone after 120 min incubation in these backgrounds, and probe **F2.2** after incubation for 120 min at 37 °C with proteinase K (ProtK) in these backgrounds. This figure demonstrates the low intrinsic fluorescence and low basal rate of F2.2 cleavage in CSF, versus high intrinsic fluorescence and prominent basal rate of F2.2 cleavage in serum.
(b) Incubation of probe **F2.2** in different backgrounds with r-$IgAP_{Hinf1}$ (2.9 μg/mL).