Protease-Resistant Peptide Ligands from a Knottin Scaffold Library

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S *Supporting Information*

ABSTRACT: Peptides within the knottin family have been shown to possess inherent stability, making them attractive scaffolds for the development of therapeutic and diagnostic agents. Given its remarkable stability to proteases, the cyclic peptide kalata B1 was employed as a scaffold to create a large knottin library displayed on the surface of *E. coli*. A library exceeding 10^9 variants was constructed by randomizing seven amino acids within a loop of the kalata B1 scaffold and screened using fluorescence-activated cell sorting to identify peptide ligands specific for the active site of human thrombin. Refolded thrombin binders exhibited high nanomolar affinities in solution and slow dissociation rates and were able to inhibit thrombin's enzymatic activity. Importantly, 80% of a knottin-based thrombin inhibitor remained intact after a 2 h incubation both with trypsin and with chymotrypsin, demonstrating that modifying the kalata B1 sequence did not compromise its stability properties. In addition, the knottin variant mediated 20-fold enhanced affinity for thrombin, when compared to the same seven residue binding epitope constrained by a single disulfide bond. Our results indicate that peptide libraries derived from the kalata B1 scaffold can yield high-affinity protein ligands that retain the remarkable protease resistance associated with the parent scaffold. More generally, this strategy may prove useful in the development of stable peptide ligands suitable for *in vivo* applications.
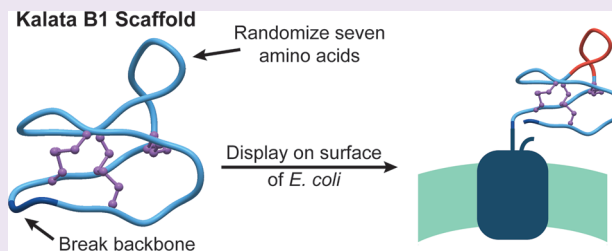
Peptides are increasingly attractive as therapeutics given their diverse biological functions and potentially high potency and target specificity.[1] Yet the development of therapeutic peptides remains challenging given their typically short *in vivo* half-life that results from rapid renal clearance and from degradation by peptidases in the blood, liver, and kidneys.[2] Susceptibility to both exo- and endopeptidases can contribute substantially to the clearance of peptides in circulation, with slow renal filtration increasing exposure to protease degradation.[3] Furthermore, peptidase susceptibility may increase the risk of immunogenicity, mediated by antigen-processing proteases.[4] Consequently, several strategies to enhance peptide resistance to proteases have been developed including N-terminal acetylation and C-terminal amidation,[5] the use of backbone cyclization,[6] incorporation of noncanonical or D-amino acids,[7,8] and PEGylation.[9] Although these approaches have proven useful alone or in combination, there remains a need for general strategies to improve half-life that are compatible with recombinant protein synthesis and preserve the benefits of small size.

An alternative strategy to enhance the intrinsic stability of peptides is the use of naturally occurring disulfide-constrained peptide scaffolds that are resistant to proteolysis. In particular, the knottin family of peptides provides attractive candidate scaffolds for the development of peptidase-resistant peptides because of their compact and stable cystine knot structure formed by three disulfide bonds.[10] Two disulfide bonds, along with the backbone, form a ring through which a third disulfide bond threads, forming the cystine knot structure. A variety of knottin family members have been utilized as scaffolds for developing stable peptide ligands

through loop grafting of bioactive peptides.[11] For example, binding epitopes were successfully grafted into a loop of *Ecballium elaterium* trypsin inhibitor II (EETI-II). The grafted binder was enriched from an excess of nonbinding peptides after surface display on *E. coli* and screening *via* fluorescence-activated cell sorting (FACS).[12] Similarly, thrombopoietin receptor binding peptides were inserted into both the Agouti-related protein (AGRP) and the EETI-II peptide scaffolds to yield potent antagonists of platelet production and when homodimerized became effective agonists.[13] The circular peptide kalata B1 was modified by replacing individual loops with a vascular endothelial growth factor binding sequence, and many of the scaffold-constrained sequences exhibited dramatically improved serum stability compared to the linear epitope.[14]

To directly identify variants selective for arbitrary targets, peptide libraries presented within disulfide-constrained scaffolds have been constructed and displayed on phage or yeast for screening. For example, randomization of a loop in the AGRP scaffold, in conjunction with yeast display library screening using FACS, was used to successfully identify $\alpha_v\beta_3$ integrin ligands with affinities in the high picomolar to low nanomolar range.[15] Kunitz-domain inhibitors are larger than knottins, having approximately 60 amino acids rather than 30 amino acids, and contain three disulfide bonds that are not connected in a knot conformation. Phage display libraries based on two different Kunitz domains,

human lipoprotein-associated coagulation inhibitor and bovine pancreatic trypsin inhibitor, have yielded ligands binding to a variety of enzyme targets including neutrophil elastase, chymotrypsin, pancreatic elastase, thrombin, plasmin, and human plasma kallikrein.[16−19] A potent plasma kallikrein inhibitor (Ecallantide) with a K_i of 44 pM was developed using this approach and approved for the treatment of hereditary angioedema.[20] These examples demonstrate the utility of disulfide-rich peptide libraries.

Despite their stabilizing cystine knot structure, many knottin peptides remain susceptible to degradation by proteases *in vivo*. Derivatives of AGRP and two squash protease inhibitors were stable to pepsin and elastase but were extensively degraded by trypsin and chymotrypsin.[21] Similarly constrained microproteins, including ω-conotoxin MVIIa and a hybrid of two squash trypsin inhibitors (EETI-II and MCoTI-II), were degraded by trypsin, with the latter also degraded by chymotrypsin.[22,23] The cyclotide kalata B1 is a remarkable member of the knottin family because it is completely resistant to trypsin and pepsin degradation for up to 24 h.[24] Furthermore, kalata B1 exhibits exceptional chemical and thermal stability, making it an attractive scaffold for therapeutic design.[24] Although native kalata B1 exhibits a circular backbone,[25] some acyclic permutants retain the stability of the parent cyclotide,[24] exhibit reduced undesired hemolytic activity, fold with the correct disulfide connectivity,[26] and are amenable to display technologies. Thus, we constructed a large bacterial display library by randomizing a single loop within kalata B1 and fusing the acyclic scaffold to an engineered bacterial display protein (eCPX).[27,28] The library was screened for binders to human thrombin, a serine protease involved in the blood coagulation cascade. Our results indicate that the kalata B1 scaffold can yield trypsin- and chymotrypsin-resistant thrombin inhibitors and may provide a general methodology to create peptide ligands with increased stability for diagnostic or therapeutic use.

RESULTS AND DISCUSSION

Design and Screening of a Knottin Library. A knottin peptide library was displayed on the cell surface of *E. coli* by opening the native cyclic structure of kalata B1 between two glycine residues within loop two and fusing the resulting kalata B1 C-terminus to the N-terminus of the eCPX display scaffold (Figure 1).[27] A fully randomized seven amino acid peptide library was created within loop six of kalata B1; we chose this loop because it exhibits a high degree of sequence variability across the cyclotide family and is the longest naturally occurring loop.[25] Transformation into *E. coli* yielded a library of 6×10^9 independent transformants, providing ~18% coverage of the theoretical library diversity (3.4×10^{10} sequence variants) based on the use of NNS codons.[29]

The kalata B1 library was enriched for α-thrombin binders using three cycles of FACS, with 30 nM biotinylated thrombin in the first two cycles, and a kinetic screen for slow dissociation rates in the third cycle.[30] The sequences of individual isolated clones revealed five distinct consensus groups (Table 1). A few of the isolated peptides had eight or nine, rather than seven, amino acids within the randomized region, likely due to PCR errors during library construction or oligonucleotide primer errors. All of the identified groups included a conserved arginine or lysine residue, whereas only two groups contained an acidic residue, aspartic acid. Two of the consensus groups had a glycine pair within the randomized region imparting more flexibility to the loop, and another group exhibited two conserved proline residues, which would add to the
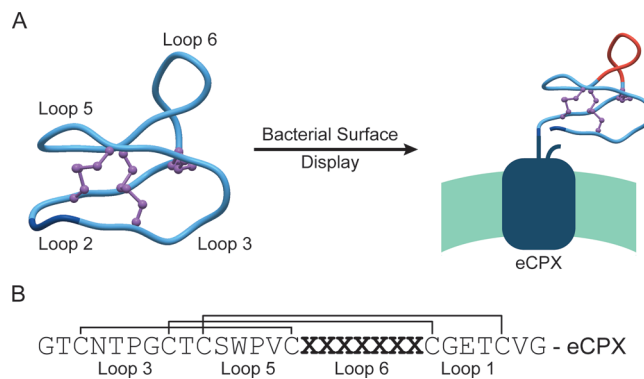

Figure 1. Construction of a library of kalata B1 variants displayed on the cell surface of *E. coli*. (A) The backbone of the cyclic kalata B1 peptide (PDB: 1NB1) was broken in loop two between two glycine residues (shown in dark blue) and fused to the display scaffold eCPX. Loop six was randomized to form the library (shown in red). (B) Sequence of the kalata B1 peptide shown with the disulfide bond connectivity, the randomized region, and loop designations.

rigidity of loop six. Clonal peptide affinities were ranked according to their apparent dissociation rates (Supplementary Figure 1), and clones with the slowest dissociation rate constants from each consensus group were characterized further.

Although kalata B1 has been utilized as a scaffold for grafting,[14] the present study, to our knowledge, is the first demonstration that large libraries constructed using the kalata B1 scaffold can rapidly yield high-affinity peptide ligands specific for a given target protein. When compared to loop grafting, the present method for ligand generation is advantageous because the library is created by loop randomization within the environment of the constrained scaffold, increasing the likelihood that the peptide will retain high affinity in solution and enabling the measurement of displayed peptide function in a structurally relevant context.

The Kalata B1 Scaffold Confers Binding Affinity. To investigate the effect of the kalata B1 scaffold upon affinity, peptides corresponding to the thrombin-binding region (loop six) of clone THR-5 were constructed as both a single-disulfide constrained peptide and as a linear peptide and displayed on *E. coli*. Peptide-displaying cells were incubated with a concentration series of α-thrombin and analyzed by flow cytometry in triplicate to measure K_D^{App} values (Figure 2). The kalata B1 scaffold, having three disulfide bonds, exhibited hindered export to the outer membrane when compared to the linear or single-disulfide constrained peptides, suggesting that periplasmic disulfide formation and/or folding may interfere with export.[31] For clone THR-5 in the kalata B1 scaffold, the K_D^{App} was calculated to be 670 nM. In contrast, the linear peptide showed negligible binding signal above background at the highest thrombin concentration tested. A single-disulfide constrained loop construct exhibited binding, but the cell fluorescence did not saturate at the highest concentration of thrombin used. However, inspection of the resulting curve suggests that the K_D^{App} of the constrained loop is greater than 15 μM. Similarly, clone THR-24 within the kalata B1 scaffold had a K_D^{App} of 320 nM, while the disulfide-constrained and linear peptides exhibited K_D^{App} values greater than 6 μM (not shown). Collectively, these results indicate that the kalata B1 scaffold imparts at least a 20-fold increase in affinity to the thrombin-binding epitope relative to a single-disulfide constrained loop. The conformational dependence of the affinity of the binding epitope highlights the importance of screening a

Table 1. Thrombin-Binding Kalata B1 Variants[a]

Clone	Sequence
THR-1	GTCNTPGCTCSWPVC**IDGGRFL**CGETCVG
THR-2	GTCNTPGCTCSWPVC**IDGGRFTWQ**CGETCVG
THR-3	GTCNTPGCTCSWPVC**IDGGRFW**CGETCVG
THR-4	GTCNTPGCTCSWPVC**IDGGRLF**CGETCVG
THR-5	GTCNTPGCTCSWPVC**IDGGRLM**CGETCVG
THR-6	GTCNTPGCTCSWPVC**IDGGRYM**CGETCVG
THR-7	GTCNTPGCTCSWPVC**IDGGRWW**CGETCVG
THR-8	GTCNTPGCTCSWPVC**IDGGRWI**CGETCVG
THR-9	GTCNTPGCTCSWPVC**IDGGRWM**CGETCVG
THR-10	GTCNTPGCTCSWPVC**VDGGRWM**CGETCVG
THR-11	GTCNTPGCTCSWPVC**LDGGRWM**CGETCVG
THR-12	GTCNTPGCTCSWPVC**LDGGRFM**CGETCVG
THR-13	GTCNTPGCTCSWPVC**VDGGRML**CGETCVG
THR-14	GTCNTPGCTCSWPVC**VDGGRMW**CGETCVG
Consensus	**I/L/V-D-G-G-R-X-X**
THR-15	GTCNTPGCTCSWPVC**MVRDRWL**CGETCVG
THR-16	GTCNTPGCTCSWPVC**MWRDRWL**CGETCVG
Consensus	**M-X-R-D-R-W-L**
THR-17	GTCNTPGCTCSWPVC**SMRFPWG**CGETCVG
THR-18	GTCNTPGCTCSWPVC**SARWPWG**CGETCVG
THR-19	GTCNTPGCTCSWPVC**SIRWPWG**CGETCVG
Consensus	**S-X-R-F/W-P-W-G**
THR-20	GTCNTPGCTCSWPVC**RVLGGYMV**CGETCVG
THR-21	GTCNTPGCTCSWPVC**RVNGGYSVV**CGETCVG
THR-22	GTCNTPGCTCSWPVC**RVQGGYTI**CGETCVG
THR-23	GTCNTPGCTCSWPVC**RLRGGYQL**CGETCVG
THR-24	GTCNTPGCTCSWPVC**RLRGGYVV**CGETCVG
THR-25	GTCNTPGCTCSWPVC**RLLGGYLV**CGETCVG
THR-26	GTCNTPGCTCSWPVC**RLRGGYLV**CGETCVG
THR-27	GTCNTPGCTCSWPVC**RLTGGYHV**CGETCVG
Consensus	**R-V/L-X-G-G-Y-X-I/L/V**
THR-28	GTCNTPGCTCSWPVC**PRPKLFL**CGETCVG
THR-29	GTCNTPGCTCSWPVC**PLPRLYP**CGETCVG
Consensus	**P-X-P-R/K-L-X-X**

[a] The randomized region is bolded with the conserved residues in each consensus group highlighted.

Figure 2. Measurement of the contribution of the kalata B1 scaffold to the apparent binding affinity for thrombin. The bacterial display clone THR-5 exhibited a $K_D^{APP} = 670$ nM, corresponding to an affinity at least 20-fold greater than that of the same peptide in a single-disulfide constrained loop conformation. The epitope as a linear peptide did not appreciably bind at any of the thrombin concentrations that were assayed.

knottin library against the desired protein target as opposed to grafting a bioactive loop into the scaffold.

Isolated Knottins Bind Thrombin's Active Site. To identify the location of the knottin binding site(s) on human thrombin, a competition binding assay was used. Peptide-displaying clones chosen from each consensus group were incubated with biotinylated α-thrombin and a concentration series of a given unlabeled competitor (Supplementary Figure 2). Thus, a decrease in cell fluorescence with increasing concentrations of competitor indicated that the competitor interfered with the binding of α-thrombin to the kalata B1 peptide. First, hirudin, which binds to both the active site and exosite I, inhibited thrombin binding, which indicates that the peptides require the active site or exosite I for interaction, rather than exosite II. Second, three direct competitors to the labeled α-thrombin, unlabeled α-thrombin, γ-thrombin (exosite I removed by proteolysis), and thrombin with the active site blocked by the inhibitor PPACK,[32] were assayed for their ability to compete with thrombin. As expected, competition with unlabeled α-thrombin caused a large decrease in cell fluorescence. Increasing concentrations of γ-thrombin caused a similar decrease in fluorescence for all of the peptides assayed, indicating that the peptides do not bind exosite I. Furthermore, when the thrombin
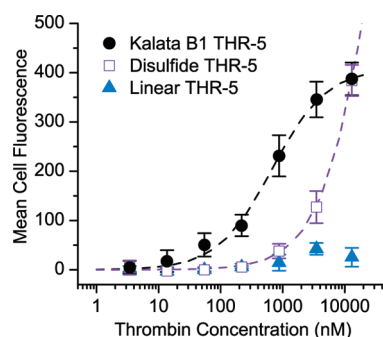
active site was preblocked by the irreversible inhibitor PPACK, the complex was not able to compete with the labeled α-thrombin. Surprisingly, however, an exosite I-binding DNA aptamer (GGTTGGTGTGGTTGG)[33] competed with the peptides. Conceivably, the DNA aptamer could allosterically alter the active site and block the peptide from interacting with thrombin.[34] Alternatively, the competition may be due to steric hindrance or poor specificity since the aptamer is able to bind to two separate thrombin molecules by interacting with exosite I and II simultaneously. Four different clones (THR-5, THR-29, THR-24, and THR-17) exhibited the same behavior in this competition assay. Collectively, these results indicate that the identified peptides bind the active site of thrombin.

Refolded Peptides Bind with Nanomolar Affinity and Inhibit Activity. To characterize their affinity, activity, and protease resistance, multiple thrombin-binding peptides were synthetically prepared and oxidized to form the characteristic cystine knot of kalata B1. The refolded peptides acyclic kalata B1, THR-5, and THR-29 were purified by RP-HPLC (Figure 3). Peptides THR-20 and THR-24, both from the same consensus group, could not be purified in their crude, reduced form due to poor solubility and therefore were not successfully refolded. Kalata B1 is hydrophobic due to large, nonpolar residues in loop five and six that become surface exposed when the cyclic cystine knot is properly formed. Consequently, scaffold engineering may be beneficial to increase the solubility and folding efficiency of acyclic kalata B1 derived ligands.

The acyclic kalata B1 refolding mixture exhibited a sharp, late-eluting peak, which has been shown to correspond to the native-like structure for kalata B1 due the surface exposure of hydrophobic residues.[26] For the refolded THR-5 mixture, the separation between the misfolded material and the large, late-eluting peak was reduced probably as a result of the randomization of loop six, which disrupted a portion of the surface exposed hydrophobic patch. Purification eliminated the observed shoulder, yielding a 90% pure product as determined *via* LC−MS. The refolded product of THR-29, however, exhibited a broad elution profile suggestive of a more heterogeneous mixture. Peptides corresponding to the indicated elution peaks for the variants (Figure 3) possessed three disulfide bonds as verified *via* mass spectrometry.

The binding kinetics of the refolded knottins were measured using surface plasmon resonance (Figure 4). Knottin THR-5
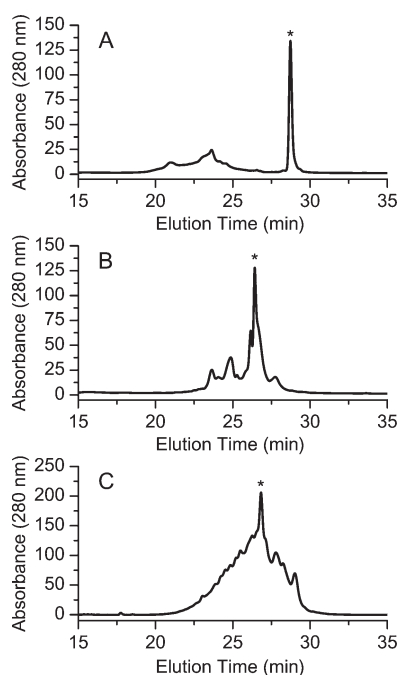
Figure 3. Purification of the refolded kalata B1 peptides. RP-HPLC absorbance traces for (A) kalata B1, (B) THR-5, and (C) THR-29. The asterisk indicates the refolded product characterized using surface plasmon resonance and protease stability assays.

Peptide	k_{on} (M^{-1}s^{-1})	k_{off} (s^{-1})	K_D (nM)	χ^2
THR-5	4.71 x 10^3	2.36 x 10^{-3}	500	0.69
THR-29	1.83 x 10^4	5.98 x 10^{-3}	330	1.63

Figure 4. Measurement of thrombin-binding knottin affinity. Response curves were measured using surface plasmon resonance for (A) THR-5 and (B) THR-29 binding to thrombin immobilized on a CM5 chip. The raw data are shown as a solid black line with the fit (1:1 Langmuir binding model) as a dashed red line. A 2-fold dilution series from 78 nM to 2.5 μM was used for each of the peptides with each series run in duplicate. The kinetic values calculated from each of the fits (association rate constant, dissociation rate constant, and K_D) are given.

exhibited slower association and dissociation rate constants when compared to clone THR-29, and the shape of the kinetic binding response closely resembled that of an antibody. The K_D values for both thrombin-binding knottins were between 300 and 500 nM. The native, acyclic kalata B1 peptide produced less than a 5 RU response at the highest peptide concentration tested (10 μM), confirming that the knottin scaffold did not appreciably bind to thrombin.

The kalata B1 scaffold conferred antibody-like binding kinetics, characterized by slow association and dissociation rates, to the discovered thrombin-binding epitopes. Interestingly, the therapeutic thrombin inhibitor bivalirudin (Angiomax) has an association rate constant 4 orders of magnitude larger than that of THR-5 and THR-29 (4 × 10^8 *versus* 10^3–10^4 M^{-1} s^{-1}), balanced by a rapid dissociation rate over 2 orders of magnitude faster (0.8 *versus* 10^{-3} s^{-1}).[35] Other constrained peptide scaffolds have been shown to exhibit similarly slow dissociation rate constants as those measured here, a feature that may prove useful for *in vivo* applications. For example, variants of Min-23, a truncated form of EETI-II, bind an anti-Epstein–Barr virus antibody with an association rate constant of 3.9 × 10^4 M^{-1} s^{-1} and a dissociation rate constant of 6.1 × 10^{-4} s^{-1}.[36]

To determine whether the soluble kalata B1 variants were effective active site inhibitors, thrombin inhibition by knottin THR-5 was measured using a chromagenic thrombin substrate. Different concentrations of the peptide were assayed with a single concentration of thrombin and substrate (Supplementary Figure 3). An IC$_{50}$ value of 8.4 ± 0.7 μM was calculated from triplicate experiments. Peptides identified here interact exclusively with the active site and achieve modest inhibitory activity. Many naturally occurring peptides achieve high affinity and inhibitory activity by bivalent binding to both the active site and exosite I of thrombin. For example, derivatives of the
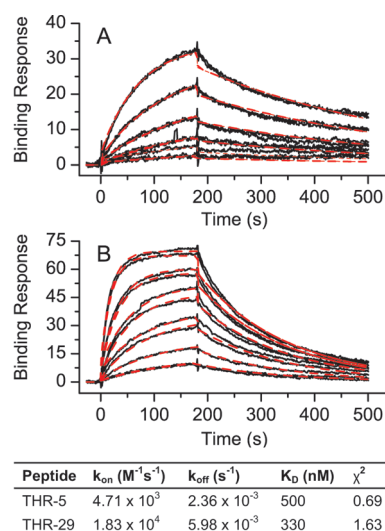
approved thrombin inhibitor hirudin have K_i values between 60 and 200 fM.[37] In addition, Kunitz-domain inhibitors isolated from ticks also exhibit high thrombin inhibitory activity with K_i values in the low picomolar to low nanomolar range.[38,39]

The affinities of the thrombin-binding knottins could be improved by randomizing an additional loop in the scaffold to generate a peptide inhibitor that mimics the bivalent binding of bivalirudin. Bivalirudin contains an active site binding motif, a short tetra-glycine linker, and an anionic peptide that interacts with exosite I. The active site inhibitor sequence in bivalirudin (D-Phe-Pro-Arg-Pro) does not significantly inhibit thrombin even at concentrations up to 10 μM,[40] while THR-5 had an IC$_{50}$ in the low-micromolar range. Furthermore, the potency of bivalirudin is reduced by slow hydrolysis within the active site binding ligand,[35] whereas the kalata B1 variants are highly resistant to proteolysis. The intact bivalirudin peptide, however, has a K_i of 2 nM,[40] illustrating the dramatic effect of a bivalent interaction for increasing inhibitory activity. A new library could be constructed based on peptide THR-5 by randomizing loop three in the scaffold[41] and then sorting with more stringent conditions to isolate an inhibitor that interacts with both exosite I and the active site to improve its affinity.

Variants Retain the Proteolytic Stability of Kalata B1. To determine whether the thrombin-binding knottins retained the proteolytic resistance characteristics of the parent kalata B1 peptide, their ability to resist degradation by trypsin (10 μg mL^{-1}) and chymotrypsin (100 μg mL^{-1}) was measured. The trypsin concentration was chosen to enable direct comparisons with previous studies characterizing the trypsin resistance of kalata B1.[24,42] With both proteases, 80% of knottin THR-5 remained intact following a 2 h incubation and exhibited dramatically enhanced resistance compared to the control peptides (Figure 5). The fraction of THR-5 that was degraded by trypsin was hydrolyzed after the arginine residue of the binding epitope
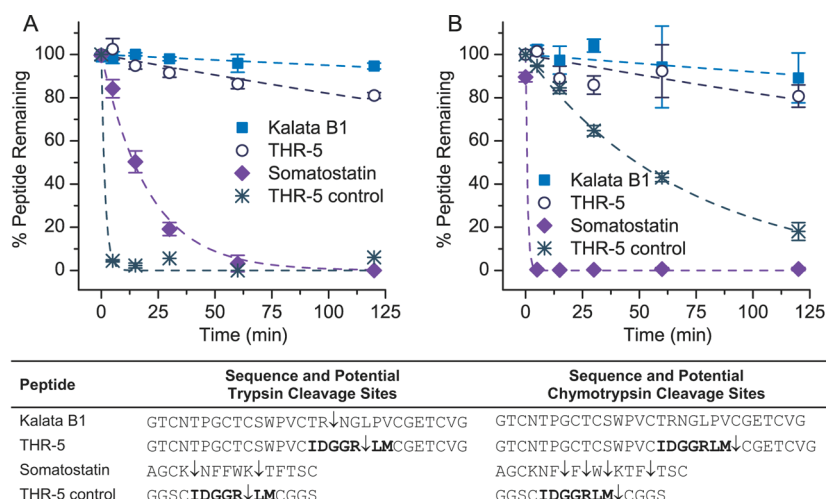
Peptide	Sequence and Potential Trypsin Cleavage Sites	Sequence and Potential Chymotrypsin Cleavage Sites
Kalata B1	GTCNTPGCTCSWPVCTR↓NGLPVCGETCVG	GTCNTPGCTCSWPVCTRNGLPVCGETCVG
THR-5	GTCNTPGCTCSWPVC**IDGGR**↓**LM**CGETCVG	GTCNTPGCTCSWPVC**IDGGRLM**↓CGETCVG
Somatostatin	AGCK↓NFFWK↓TFTSC	AGCKNF↓F↓W↓KTF↓TSC
THR-5 control	GGSC**IDGGR**↓**LM**CGGS	GGSC**IDGGRLM**↓CGGS

Figure 5. Trypsin and chymotrypsin resistance of a thrombin-specific knottin THR-5. The acyclic kalata B1 parent, THR-5, somatostatin, and a cyclic, control peptide corresponding to the binding loop of THR-5 were incubated with (A) trypsin and (B) chymotrypsin and analyzed by LC−MS. The sequences for each of the peptides are listed along with the potential cleavage sites. Kalata B1 was stable over the 2 h incubation period with both proteases, and 80% of THR-5 remained intact. Somatostatin and the THR-5 control were readily degraded by both trypsin and chymotrypsin. The curves were fit to a second-order reaction model, and the experiments were performed in duplicate.

in loop six. Chymotrypsin cleaved after the methionine residue also found in the binding loop of THR-5. The acyclic kalata B1 peptide did not show appreciable degradation by trypsin or chymotrypsin over the entire 2 h incubation as previously observed.[24]

In contrast, the THR-5 binding loop constrained by a single disulfide bond was completely degraded in less than 5 min by trypsin. In fact, the majority of the degradation occurred within 1 min following trypsin addition before the reaction could be fully quenched. Furthermore, an unrelated trypsin-sensitive peptide containing two lysines (somatostatin 14) was rapidly degraded by trypsin. Somatostatin, which is also constrained by a single disulfide bond, was completely cleaved by trypsin at both cleavage sites within 1 h. By fitting the peptide degradation curves to a second-order reaction model, THR-5 in the kalata B1 scaffold is degraded by trypsin at least 100-fold more slowly than the THR-5 epitope constrained by a single disulfide bond.

The two control peptides were also extensively degraded by chymotrypsin. The THR-5 loop control was cleaved following the methionine residue and less than 20% of the intact peptide remained after the 2 h incubation. The THR-5 control peptide was degraded 8-fold faster than the kalata B1 variant as determined by the ratio of the second-order rate constants. In addition, somatostatin, which contains multiple large, hydrophobic residues that are the preferred substrates for chymotrypsin, was cleaved at two or more sites within 5 min and was extensively degraded at all four cleavage sites within 2 h. Thus, these results demonstrate that THR-5 was significantly more stable than the single-disulfide constrained control peptides, and its proteolytic resistance approached that of the acyclic, parental kalata B1 molecule.

Trypsin and chymotrypsin were chosen for the stability assay because THR-5 had accessible cleavage sites in the thrombin-binding region (loop six) of the peptide. Because trypsin and chymotrypsin are representative members of large classes of proteases and have high activity combined with broad substrate specificity, they are commonly used to assess the relative proteolytic stability of peptides.[21,24] The stability of THR-5 is

notable since other disulfide-constrained peptides are susceptible to trypsin and chymotrypsin degradation. The cystine knot structure of native kalata B1 is believed to be critical for its proteolytic stability. Although the use of NMR is generally required to verify the disulfide connectivity,[25] the observations that (i) the elution profile for refolded THR-5 contained a large, late-eluting peak indicative of surface exposure of a hydrophobic patch and (ii) THR-5 exhibited a trypsin resistance comparable to that of the acyclic permutant of kalata B1 suggested that THR-5 possessed a native-like cystine knot structure.[26] In contrast, THR-29 exhibited multiple elution peaks corresponding to distinct peptide conformers constrained by three disulfide bonds. The prolines present in loop six of THR-29 may have hindered its folding into the native conformation. In addition to the cystine knot, backbone N- to C-terminal cyclization could further enhance the stability of THR-5 and other kalata B1 derived peptides by increasing resistance to exopeptidases.[43]

Conclusions. In summary, a peptide library derived from the stable kalata B1 scaffold was successfully screened using FACS to yield a diverse set of thrombin binders that could be grouped into multiple distinct families with unique consensus motifs. In addition, bacterial display proved to be a highly effective tool for the generation and quantitative screening of a large library of knottin peptides. While phage display, and to a lesser extent mRNA and DNA display methods, have been applied to identify and/or affinity mature peptides, relatively few reports have described the screening of peptide libraries for novel specificities using yeast or bacterial display methods.[44,45] These cell surface display technologies are advantageous because they enable quantitative library screening and clone characterization using flow cytometry. The use of the engineered display protein eCPX provided sufficient display levels, although lower than the linear and loop constructs, for screening peptide libraries derived from a complex knottin structure with multiple disulfide bonds. The reduced export of the kalata B1 scaffold did not interfere with the selection of thrombin inhibitors using bacterial display. Although reduced display can decrease the dynamic range of the mean cell fluorescence during screening, low display levels proved useful to

avoid avidity effects. Cytometry accurately reported affinities of surface displayed thrombin binders that were within 2-fold of the corresponding values measured in solution using surface plasmon resonance. This result contrasts with the poor agreement between cell surface and solution affinities observed for homomultimeric targets, where avidity and rebinding effects can skew apparent affinities,[27,28] indicating that the reduced display level may be advantageous. The kalata B1 scaffold also conferred dramatically increased binding affinity and protease resistance when compared to a single-disulfide constrained peptide. The kalata B1 variant THR-5 exhibited more than a 100-fold increased resistance to trypsin when compared to the epitope constrained by a single disulfide bond. Thus, bacterial displayed knottin libraries coupled with quantitative library screening *via* FACS provide a highly effective tool for the discovery and engineering of protease resistant peptide ligands.

■ METHODS

Bacterial Strains, Plasmids, and Growth Conditions. All bacterial display experiments utilized the plasmid pBAD33 (Cm^r) in *E. coli* strain MC1061.[46,47] Cells were subcultured 1:50 from an overnight culture into LB medium supplemented with 34 μg mL^{-1} chloramphenicol and incubated at 37 °C for 2 h with vigorous shaking. To achieve surface display, protein synthesis was induced with 0.04% (w/v) L-arabinose and the cells were incubated at 37 °C for an additional 45 min.

Construction and Screening of a Kalata B1 Library. A library of kalata B1 variants was constructed by breaking the native cyclic backbone within loop two and fusing the resulting C-terminal glycine to the N-terminus of the eCPX display scaffold.[27] The display scaffold eCPX utilizes the native OmpX signal sequence, which is cleaved during translocation of the fusion protein to the periplasm. The seven amino acids in loop six of kalata B1 were randomized using NNS degenerate codons to create a library. Assembly PCR was performed using KOD Hot Start DNA polymerase (Novagen) with overlapping primers (Supplementary Table 1, Operon). An additional PCR reaction was used to construct the library with the assembled forward primer, GGCTGAAAATCTTCTCTC as the reverse primer, and pBAD33-eCPX as the template. The PCR product and vector were digested with SfiI (New England Biolabs), ligated, and electroporated into *E. coli* MC1061, yielding 6 × 10^9 transformants.

The knottin library was sorted using one round of magnetic-activated cell sorting (MACS) followed by three cycles of FACS (BD FACSAria), as described elsewhere.[48] Human α-thrombin (Haematologic Technologies or Sigma Aldrich) was first biotinylated using the FluoReporter mini-biotin-XX protein labeling kit (Invitrogen). MACS was performed using 30 nM biotinylated α-thrombin and MyOne streptavidin-coated magnetic beads (Invitrogen) with a bead:cell ratio of 1:100. For the first two rounds of FACS, the cells were incubated with 30 nM biotinylated α-thrombin on ice for 45 min and labeled with streptavidin-R-phycoerythrin (SAPE, Invitrogen) at a final concentration of 17 nM. A third cycle of kinetic screening was performed by first incubating the population with α-thrombin (80 nM). The cells were then pelleted by centrifugation and incubated with 50 μM DNA aptamer competitor in PBS (GGTTGGTGTGGTTGG, Operon) at RT for 1 h.[49] The cells were labeled with neutravidin-R-phycoerythrin (NAPE, Invitrogen) on ice prior to sorting. NAPE was used instead of SAPE for this last round of sorting to disfavor the enrichment of streptavidin-binding peptides. Individual clones from the last two rounds of selections were isolated by plating, and the composition of the corresponding displayed peptide was determined by plasmid DNA sequencing.

Cell Surface Characterizations. To verify that the isolated clones did not bind the labeling reagents, they were first assayed for binding to

SAPE and NAPE. The dissociation rate for each clone was then measured by labeling cells with biotinylated α-thrombin at a final concentration of 800 nM, washing the cells with PBS, and incubation at RT. At specific time intervals, aliquots were removed and labeled with SAPE for cytometric analysis. To avoid rebinding effects, an induction time of 15 min was used to reduce the number of peptides on the cell surface.

The thrombin-binding epitope (*i.e.*, randomized loop) of clone THR-5 was constructed as both linear and single-disulfide constrained peptides on the cell surface by recombinantly fusing them to the N-terminus of eCPX. Cells were labeled with 4-fold serial dilutions of biotinylated α-thrombin (3.4 nM to 14 μM) and SAPE, and then analyzed by flow cytometry. The apparent equilibrium dissociation constant (K_D^{App}) was calculated by fitting the plot of mean cell fluorescence *versus* thrombin concentration to the equation

$$FL - FL_{bg} = \frac{(FL_{max} - FL_{bg})[T]}{[T] + K_D^{App}}$$

where FL is the mean cell fluorescence, FL_{max} is the maximum observed fluorescence, FL_{bg} is the background cell fluorescence of unlabeled bacteria, and $[T]$ is the thrombin concentration.[30]

To determine the thrombin-binding site of each of the consensus groups, peptides were labeled with biotinylated thrombin (875 nM) and a concentration series (5 nM to 20 μM) of unlabeled competitors including α-thrombin, γ-thrombin, PPACK-blocked thrombin (all forms of thrombin from Haematologic Technologies), hirudin (Hyphen Biomed), and a thrombin specific DNA aptamer (Operon).[49]

Refolding of the Synthetic Kalata B1 Peptides. Synthetic, reduced knottin peptides (Biomatik) were purified using RP-HPLC (HP 1100) on a Grace Vydac C18 column. The purified material was then added to the refolding buffer (1:1 0.1 M ammonium bicarbonate/isopropyl alcohol, 5 mM reduced glutathione, 0.5 mM oxidized glutathione) until the peptide concentration reached 50 μg mL^{-1}.[50] The refolding reaction was stirred at 4 °C for 70 h and then acidified to pH 3. The peptides were loaded onto a C18 column and purified using a gradient of 5—51% acetonitrile over 36 min at a flow rate of 4 mL min^{-1}. The refolded, acyclic kalata B1 mixture showed a sharp elution peak at 28 min, whereas the misfolded and partially folded kalata B1 material eluted earlier as a broad peak. Clone THR-5 also showed a clear elution peak (27 min) following the peaks containing misfolded material, although the separation between the two species was not as distinct as with kalata B1. The misfolded material contained a mixture of species with both two and three disulfide bonds present. Clone THR-29, however, did not have a clear, late eluting peak. Peptides THR-20 and THR-24 exhibited poor solubility, which prevented successful refolding. All collected fractions were analyzed using mass spectrometry on a Micromass QTOF2 ESI/TOF instrument to determine contents and degree of disulfide connectivity. In addition, the purified peaks were run on an analytical C18 column to determine peptide purity, which was calculated to be over 90% for THR-5 and acyclic kalata B1.

Affinity Measurements Using Surface Plasmon Resonance. The solution affinities of the refolded peptides were measured using a Biacore 3000 surface plasmon resonance instrument (GE Healthcare). On a CM5 chip, α-thrombin was immobilized through amine coupling. The surface was activated with a 1:1 (v:v) mixture of 0.4 M EDC (N-ethyl-N′-(3-dimethylaminopropyl)carbodiimide) and 0.1 M NHS (N-hydroxysuccinimide). Then 2 μg mL^{-1} α-thrombin in 10 mM sodium acetate buffer, pH 5, was immobilized to a level of approximately 1600 RU. Unreacted carboxyl groups were deactivated using 1 M ethanolamine, pH 8.5. An additional reference flow cell underwent the same immobilization procedure without the α-thrombin step. A kinetic binding analysis was performed using 2-fold serial dilutions of the refolded peptides (78 nM to 2.5 μM) with each of the series run in duplicate. The peptides were injected for 3 min at 50 μL min^{-1} followed

by a 6 min dissociation period with HBS-EP (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05% (v/v) Tween 20, pH 7.4) as the running buffer. A regeneration step of 10 mM sodium acetate, pH 5, injected for 12 s at 100 μL min^{-1} was performed after each injection of THR-5, whereas only a single buffer injection was required for the regeneration of THR-29. Response signals were double referenced by subtracting the signals of both a reference flow cell as well as blank buffer injections. BIAevalution software (GE Healthcare) was used to calculate the equilibrium dissociation constant (K_D) values by fitting the data to a 1:1 Langmuir binding model.

The response curves did not exactly fit the chosen 1:1 model with the most deviation occurring at high peptide concentrations. To test whether other artifacts may be causing the different shape of the curves, mass transfer limitations and linked reactions were tested using the Biacore wizards. Mass transfer was not a major effect as established by the constant slope of the association curve with increasing flow rates (15, 30, 75 μL min^{-1}). To determine if there were multiple species present in the analyte or if a conformational change occurred during the binding, the peptide was injected for increasing lengths of time and the shape of the dissociation curves were compared. The overlaying dissociation curves resulting from this test indicated that the analyte did not contain more than one binding species.

Inhibition of Thrombin by Kalata B1 Variants. To determine if the thrombin-binding knottins could inhibit the enzymatic activity of thrombin, the peptides were tested with a chromogenic peptide substrate and α-thrombin. A concentration series of refolded THR-5 (470 nM to 18.3 μM) was preincubated with 2 nM α-thrombin (4360 U mg^{-1}) in the following buffer (50 mM Tris-HCl, 150 mM NaCl, 2.5 mM CaCl$_2$, pH 8) for 45 min. The assay was initiated by adding 25 μM chromogenic peptide substrate (Pefachrome TH, Pentapharm) and was run at 25 °C. The IC$_{50}$ was calculated by fitting the normalized initial velocities *versus* inhibitor concentration to the Langmuir isotherm equation.[51]

Trypsin and Chymotrypsin Stability Assays. For the trypsin digests, the refolded peptides (final concentration of 0.5 mg mL^{-1} in HBS-EP buffer) were mixed with 0.1 mg mL^{-1} trypsin (Sigma Aldrich, 12,500 BAEE U mg^{-1}) to a final peptide:trypsin mass ratio of 50:1 and incubated at 37 °C.[24] Similarly, chymotrypsin (Thermo Scientific, 56 BTEE U mg^{-1}) was added to the peptides (in HBS buffer with no EDTA or Tween) at a final peptide:chymotrypsin mass ratio of 5:1 and kept at 37 °C. Two control peptides, somatostatin 14 (AGCKNFFW-KTFTSC, Anaspec) and clone THR-5 as a single-disulfide constrained peptide (GGSCIDGGRLMCGGS, Biomatik), were used as positive controls for the trypsin and chymotrypsin digestions. Following the addition of the protease, aliquots were removed at 0, 5, 15, 30, 60, and 120 min and the digestion quenched by the addition of TFA to a final concentration of 0.1% (v/v). Peptides were analyzed by LC−MS (Waters 2695 HPLC system/Micromass QTOF2) with a gradient of 5−46% acetonitrile over 32 min and recovery was calculated by integration of the 280 nm signal. To determine relative cleavage rates of the peptides, the stability data (% peptide remaining *versus* time) were fit to the following second-order rate model:

$$\%\text{peptide remaining} = 100 \times e^{-(k_{cat}/K_m)[E]t}$$

where k_{cat}/K_m is the rate constant, t is time, and $[E]$ is the enzyme concentration, which is assumed constant, resulting in the kinetics following a pseudo-first-order form.[52] The ratio of the rate constants for two different peptides gave a relative measure of their stability.

ASSOCIATED CONTENT

Ⓢ Supporting Information. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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ABBREVIATIONS USED

FACS, fluorescence-activated cell sorting; MACS, magnetic-activated cell sorting; eCPX, enhanced circularly permuted OmpX; EETI-II, *Ecballium elaterium* trypsin inhibitor II; AGRP, Agouti-related protein; K_D^{App}, apparent equilibrium dissociation constant; K_D, equilibrium dissociation constant; RP-HPLC, reverse-phase high performance liquid chromatography

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