

Biosynthesis and Antimicrobial Evaluation of Backbone-Cyclized α -Defensins

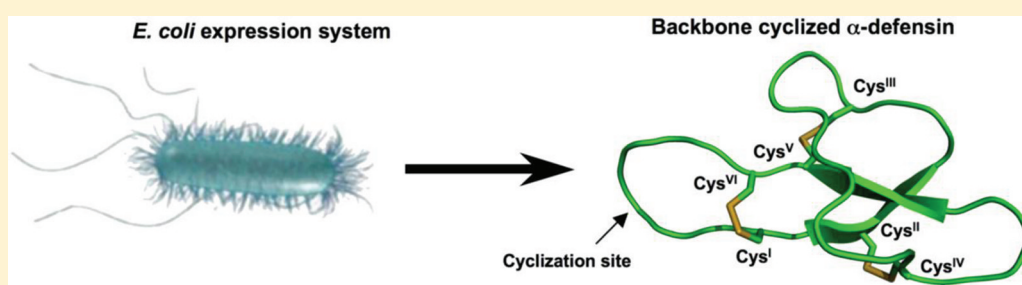
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S Supporting Information



ABSTRACT: Defensins are antimicrobial peptides that are important in the innate immune defense of mammals. Upon stimulation by bacterial antigens, enteric α -defensins are secreted into the intestinal lumen where they have potent microbicidal activities. Cryptdin-4 (Crp4) is an α -defensin expressed in Paneth cells of the mouse small intestine and the most bactericidal of the known cryptdin isoforms. The structure of Crp4 consists of a triple-stranded antiparallel β -sheet but lacks three amino acids between the fourth and fifth cysteine residues, making them distinct from other α -defensins. The structure also reveals that the α -amino and C-terminal carboxylic groups are in the proximity of each other ($d \approx 3 \text{ \AA}$) in the folded structure. We present here the biosynthesis of backbone-cyclized Crp4 using a modified protein splicing unit or intein. Our data show that cyclized Crp4 can be biosynthesized by using this approach both in vitro and in vivo, although the expression yield was significantly lower when the protein was produced inside the cell. The resulting cyclic defensins retained the native α -defensin fold and showed equivalent or better microbicidal activities against several Gram-positive and Gram-negative bacteria when compared to native Crp4. No detectable hemolytic activity against human red blood cells was observed for either native Crp4 or its cyclized variants. Moreover, both forms of Crp4 also showed high stability to degradation when incubated with human serum. Altogether, these results indicate the potential for backbone-cyclized defensins in the development of novel peptide-based antimicrobial compounds.

The cyclization of peptides has commonly been used to generate more active and stable scaffolds for therapeutic purposes.^{1–6} In general, backbone-cyclized peptides are more resistant than linear peptides to chemical, thermal, and enzymatic degradation.⁷ Because circular peptides have no N- and C-termini, they are more rigid, endowing them with the ability to persist in physiologic environments such as blood serum.^{3,6}

The production of backbone-cyclized or circular peptides can be performed chemically by using solid-phase peptide synthesis in combination with native chemical ligation^{8–13} or recombinantly in bacteria by using modified protein splicing units or inteins.^{14–16} The latter method has been used for the biosynthesis of several disulfide-rich cyclic peptides such as cyclotides^{17,18} and sunflower trypsin inhibitor 1 (SFTI-1).¹⁹ The expression of cyclic peptides in vivo has many applications, including the generation of peptide libraries for high-throughput screening of biological activities such as antimicrobial activity or

specific inhibition of protein–protein interactions involved in various pathologies.

Mammalian defensins are a family of disulfide-stabilized, host defense peptides.^{20–24} They are classically known for their antimicrobial activities but play a role in many other defense mechanisms, including wound healing,^{25,26} immune modulation,^{27–30} neutralization of endotoxin,^{31–33} and anticancer activities.³⁴ Mammalian defensins are cationic peptides with largely β -sheet structures and six conserved cysteines, which form three intramolecular disulfide bonds (Figure 1). They are divided into three structurally distinct groups, α -, β -, and θ -defensins. The overall fold of α - and β -defensins is quite similar despite differences in disulfide connectivities, and the presence of an N-terminal α -helix segment in β -defensins that is lacking

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defensins to improve biological activity while providing excellent stability to serum degradation, highlighting the potential of these peptide scaffolds for the development of novel antimicrobial compounds.

EXPERIMENTAL PROCEDURES

Analytical reverse-phase HPLC (RP-HPLC) was performed on an HP1100 series instrument with 220 and 280 nm detection using a Vydac C4 or C18 column (5 mm, 4.6 mm × 150 mm) at a flow rate of 1 mL/min. Semipreparative RP-HPLC was performed on a Waters Delta Prep system fitted with a Waters 2487 ultraviolet–visible (UV–vis) detector using a Vydac C4 column (5 μ m, 10 mm × 250 mm) at a flow rate of 5 mL/min. All runs used linear gradients of 0.1% aqueous trifluoroacetic acid (TFA, solvent A) versus 0.1% TFA and 90% MeCN in H₂O (solvent B). UV–vis spectroscopy was conducted on an Agilent 8453 diode array spectrophotometer. Electrospray mass spectrometry (ES-MS) was performed on an Applied Biosystems API 3000 triple quadrupole mass spectrometer. Calculated masses were obtained by using ProMac version 1.5.3. Protein samples were analyzed on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) 4 to 20% Tris–glycine gels (Lonza, Rockland, ME). The gels were then stained with Pierce (Rockford, IL) Gelcode Blue, photographed and digitized using a Kodak (Rochester, NY) EDAS 290 instrument, and quantified using NIH Image-J (<http://rsb.info.nih.gov/ij/>). DNA sequencing was performed by the DNA Sequencing and Genetic Analysis Core Facility at the University of Southern California using an ABI 3730 DNA sequencer, and the sequence data were analyzed with DNASTar (Madison, WI) Lasergene version 8.0.2. All chemicals were obtained from Sigma-Aldrich (Milwaukee, WI) unless otherwise indicated.

Cloning and in Vitro Expression of Backbone-Cyclized Crp4 Variants. Synthetic DNA oligos (Integrated DNA Technologies, Coralville, IA) encoding the different backbone-cyclized Crp4 analogues (Table S1 of the Supporting Information) were annealed and ligated into the pTXB1 vector (New England Biolabs, Ipswich, MA) using the NdeI and SapI restriction sites as described previously.^{17,49} The resulting plasmids were transformed into either BL21(DE3) or Origami2(DE3) cells (EMD Chemicals, Gibbstown, NJ) and grown in LB broth. Transformed BL21(DE3) cells were induced with 0.3 mM IPTG for 4 h at 30 °C and transformed Origami2(DE3) cells with 0.1 mM IPTG for 20 h at 22 °C. Cells were lysed in 0.1 mM EDTA, 1 mM PMSF, 50 mM sodium phosphate, and 250 mM sodium chloride buffer (pH 7.2) containing 5% glycerol by sonication. The soluble fraction was incubated with chitin beads (New England Biolabs) for 1 h at 4 °C, and the beads were washed with column buffer [0.1 mM EDTA, 50 mM sodium phosphate, and 250 mM sodium chloride buffer (pH 7.2)] containing 0.1% Triton X-100 followed by washes with column buffer without Triton X-100. The peptide was cyclized and folded in vitro using column buffer at pH 7.2 containing 50–100 mM reduced glutathione (GSH) for 2–3 days at room temperature with gentle rocking. We found that under these conditions backbone-cyclized Crp4 variants bind strongly to the chitin column and therefore were eluted using 8 M GdmCl in water. The corresponding supernatant and washes were pooled, and the backbone-cyclized Crp4 peptides were purified by semipreparative HPLC using a linear gradient from 20 to 40% solvent B over 30 min. Purified products were characterized by HPLC and ES-MS. All Crp4 variants were quantified by UV–vis using a molar

absorption coefficient of 3365 M^{−1} cm^{−1}. The expression of Crp4–intein fusion precursors was quantified by first desorption of the proteins from an aliquot of chitin beads using 8 M GdmCl and then measurement by UV–vis using a molar absorption coefficient of 39015 M^{−1} cm^{−1}.

Preparation of Crp4-R/A. The linear reduced and alkylated Crp4 (Crp4-R/A) was produced by expression of the Crp4-1 intein precursor in BL21(DE3) cells using the induction conditions described above. After purification of the fusion with chitin beads, Crp4-1 was cleaved from the intein using 100 mM NH₂OH in water at pH 7.2 for 18 h at room temperature. The resulting linear Crp4-1 peptide was reduced with 5 mM DTT at 37 °C for 3 h and alkylated with 12.5 mM iodoacetamide for 10 min at room temperature. Crp4-R/A was purified by semipreparative HPLC as described above. The purified product was characterized by HPLC and ES-MS (Figure S7 of the Supporting Information).

Expression of Native Crp4-wt and Crp4-6C/A. Native Crp4-wt and Crp4-6C/A were expressed in *E. coli*, purified, and refolded as previously described.^{50,51}

Expression of ¹⁵N-Labeled Backbone-Cyclized Crp4 Variants. Expression was conducted using BL21(DE3) cells as described above except they were grown in M9 minimal medium containing 0.1% ¹⁵NH₄Cl as the nitrogen source.^{18,52} Cyclization and folding were performed as described above. The ¹⁵N-labeled backbone-cyclized Crp4 defensins were purified by semipreparative HPLC as described above. Purified products were characterized by HPLC and ES-MS (Figure S3 of the Supporting Information).

In Vivo Expression of Crp4-1. Origami2(DE3) cells transformed with the plasmid encoding the intein precursor of Crp4-1 were induced, harvested, and lysed as described above. The insoluble pellet was first washed three times with column buffer containing 0.1% Triton X-100 and then twice with just column buffer. The resulting pellet was dissolved in a minimal amount of 8 M GdmCl in water. Both the soluble cell lysate and the solubilized cell lysate pellet were extracted using C18 SepPak cartridges (Waters, Milford, MA) with elution in a MeCN/H₂O mixture (3:2, v/v) containing 0.1% TFA. The samples were analyzed by HPLC and tandem mass spectrometry (HPLC–MS/MS) using a C18-HPLC column (5 mm, 2.1 mm × 100 mm), and H₂O/MeCN buffers containing 0.1% formic acid as the mobile phase. Typical analysis used a linear gradient from 0 to 90% MeCN in H₂O over 10 min. Detection was performed on an API 3000 ES-MS instrument using a multiple-reaction monitoring (MRM) mode. Data were collected and processed using Analyst (Applied Biosystems). The calibration curve using purified cyclic Crp4-1 was found to be linear in the range of 5–50 ng.

NMR Spectroscopy. NMR samples were prepared by dissolving ¹⁵N-labeled backbone-cyclized Crp4 variants in 80 mM potassium phosphate in a 90% H₂O/10% ²H₂O mixture (v/v) or 100% D₂O to a concentration of approximately 0.2 mM with the pH adjusted to 4.5 or 6.0 by addition of dilute HCl. All ¹H NMR data were recorded on a Bruker Avance II 700 MHz spectrometer equipped with a cryoprobe. Data were acquired at 27 °C, and 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was used as an internal reference. All three-dimensional (3D) experiments, ¹H{¹⁵N} TOCSY-HSQC and ¹H{¹⁵N} NOESY, were performed according to standard procedures⁵³ with spectral widths of 12 ppm in the proton dimension and 35 ppm in the nitrogen dimension. The carrier frequency was centered on the water signal, and the solvent was suppressed by

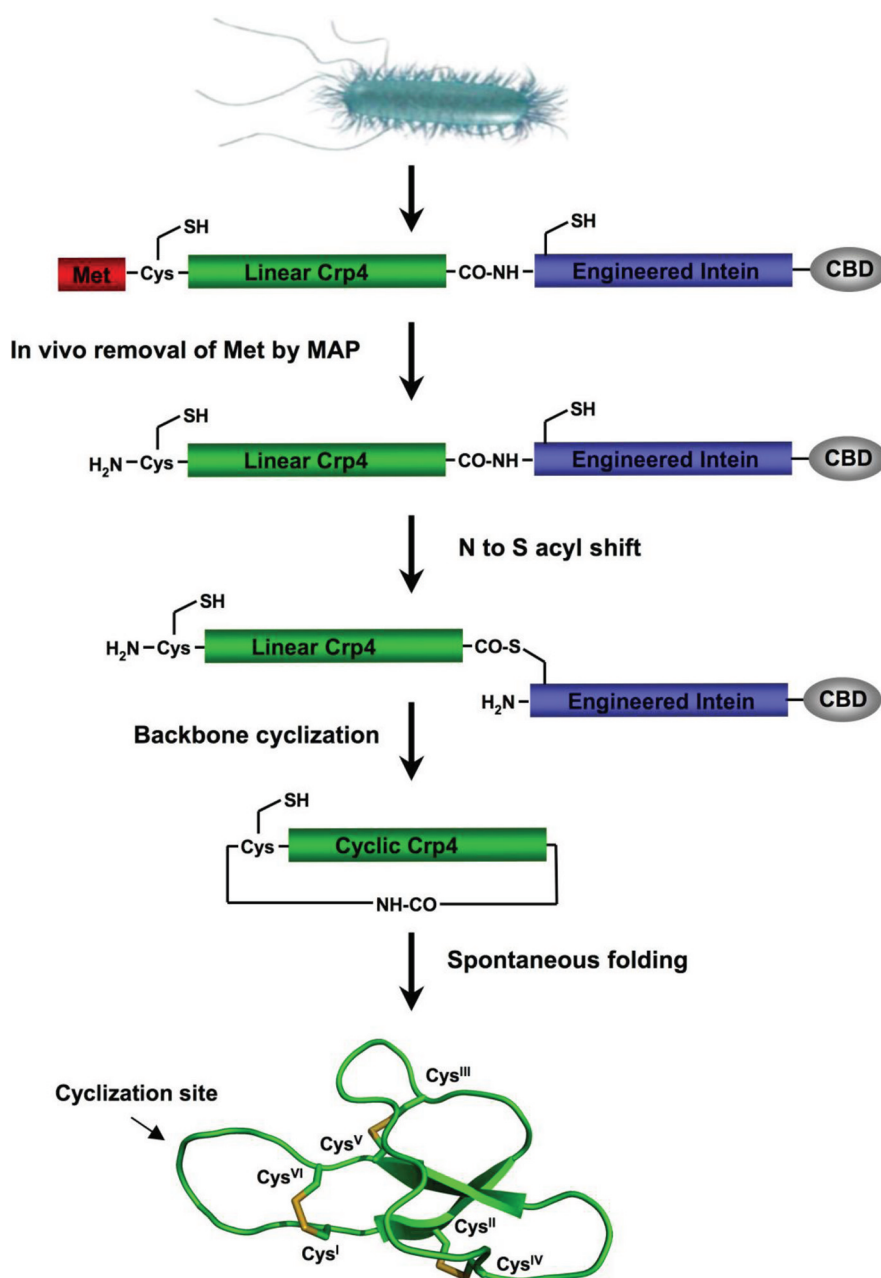


Figure 2. Biosynthesis of backbone-cyclized Crp4 variants using a modified intein in combination with native chemical ligation. CBD stands for chitin binding domain.

using a WATERGATE pulse sequence. TOCSY (spin-lock time of 80 ms) and NOESY (mixing time of 150 ms) spectra were recorded using 1024 t_3 points, 256 t_2 blocks, and 128 t_1 blocks of 16 transients. Spectra were processed using Topspin 1.3 (Bruker). Each 3D data set was apodized by a 90°-shifted sine bell-squared process in all dimensions and zero-filled to 1024 × 512 × 256 points prior to Fourier transformation. Assignments for the backbone nitrogens, H^α and H^β protons (Figures S4 and Tables S2 and S3 of the Supporting Information), were obtained using standard procedures.^{53,54}

Bactericidal Peptide Assays. Native Crp4 (Crp4-wt) and backbone-cyclized Crp4 variants were tested for bactericidal peptide activity against Gram-negative and Gram-positive bacteria. Bacteria growing exponentially in trypticase soy broth (TSB) were centrifuged at 10000g for 3 min and washed three times with 10 mM PIPES buffer (pH 7.4) supplemented

with 1% (v/v) TSB (10 mM PIPES-TSB). Approximately 1–5 × 10⁶ colony-forming units (CFU) per milliliter of bacteria were incubated with peptides in a total volume of 50 μL of 10 mM PIPES-TSB. In assays performed in the presence of salt or serum, sample mixtures were incubated in 10 mM PIPES-TSB supplemented with sodium chloride or heat-inactivated human serum, respectively. Mixtures of bacteria and peptide were incubated at 37 °C with shaking for 1 h, and 20 μL aliquot samples were diluted in 2 mL of 10 mM PIPES buffer (pH 7.4) and plated on TSB plates using an Autoplate 4000 instrument (Spiral Biotech Inc., Bethesda, MD). After incubation overnight at 37 °C, bacterial cell survival was assessed by counting CFUs.

Hemolysis Assay. EDTA-anticoagulated human blood was obtained from a healthy donor (Bioreclamation, LLC, Hicksville, NY) and centrifuged at 234g for 10 min at 22 °C. Red blood cells (RBCs) were washed four times with Dulbecco's

phosphate-buffered saline (DPBS) (Mediatech Inc., Manassas, VA) containing 4 mM EDTA and resuspended in DPBS without EDTA. Peptides diluted in DPBS to the concentrations shown were assayed for hemolysis in triplicate by incubation with 2% (v/v) RBCs for 1 h at 37 °C in an atmosphere of 5% CO₂. The cells were centrifuged at 234g for 10 min at 22 °C, and the absorbencies of the supernatants were measured at 405 nm. The hemolytic activity of each peptide was calculated relative to the 100% hemolysis obtained by incubation of RBCs with 1% Triton X-100.

Serum Stability Assay. Peptides at 100 µg/mL (~27 µM) in human serum (Lonza) were incubated at 37 °C. After various time points, triplicate serum aliquots were removed, quenched with 6 M urea in water, and incubated for 10 min at 4 °C. Subsequently, serum proteins were precipitated with 20% trichloroacetic acid for 10 min at 4 °C and centrifuged at 13000 rpm for 10 min at 4 °C. The supernatants were analyzed by C4 RP-HPLC, and the pellets were dissolved in 8 M GdmCl and also analyzed by HPLC. The percentage of peptide recovery was determined by integration of the HPLC peaks at 220 nm. Peptide identity was also confirmed by ES-MS.

RESULTS

Design of Backbone-Cyclized Crp4 Defensins. The biosynthesis of backbone-cyclized Cys-rich polypeptides using modified protein-splicing units or inteins has previously been demonstrated for several cyclotides^{17,18,49,52} and sunflower trypsin inhibitor 1 (SFTI-1).⁵⁵ Here, we have used a similar strategy for the biosynthesis of several backbone-cyclized Crp4 variants in *E. coli* cells. This approach makes use of a modified intein in combination with an intramolecular native chemical ligation reaction (NCL) (Figure 2).^{17,49} Intramolecular NCL requires the presence of an N-terminal Cys residue and a C-terminal α-thioester group in the same linear precursor molecule.^{8,14} For this purpose, the corresponding Crp4 linear precursor was fused in frame at the N- and C-termini to a Met residue and a modified *Mxe* Gyrase A intein, respectively. This allows the generation of the required C-terminal thioester and N-terminal Cys residue after *in vivo* processing by endogenous Met aminopeptidase (MAP) (Figure 2). We designed five linear Crp4 precursors (Crp4-1–Crp4-5) to explore the best ligation site as well as the best linker required for optimal cyclization and folding of the resulting backbone-cyclized Crp4 variants (Scheme 1 and Table 1). We first decided to link

Scheme 1. Sequences of the Different Crp4–Intein Precursors Used in This Study^a

Intein precursor	Sequence
Crp4-1	CYCRKGHCCKRGERVRGTCGIRFLYCCPRRGLL-intein
Crp4-2	CYCRKGHCCKRGERVRGTCGIRFLYCCPRRGLL-intein
Crp4-3	CYCRKGHCCKRGERVRGTCGIRFLYCCPRRGLL-intein
Crp4-4	CRKGHCCKRGERVRGTCGIRFLYCCPRRGLLCY-intein
Crp4-5	CCPRRGLLCYCRKGHCCKRGERVRGTCGIRFLY-intein

^aThe first three residues located at the N-terminus of Crp4-wt are underlined in blue for reference. The residues added to the ligation site to facilitate cyclization are colored green.

together through a peptide bond the native N- and C-terminal residues of Crp4-wt, Gly1 and Arg32, respectively. These two residues are in the proximity of each other in the folded structure of Crp4-wt, and in principle, the formation of this bond should not introduce too much conformational stress in

Table 1. Amino Acid Sequences of the Crp4 Peptides Used in This Study

Peptide	Sequence
Crp4-wt	GLLCYCRKGHCCKRGERVRGTCGIRFLYCCPRR ^a
Crp4-6C/A	GLL ^a YARKGHA ^b KRGERVRGTA ^b GIRFLYAA ^b PRR ^b
Crp4-1	cyclo[CYCRKGHCCKRGERVRGTCGIRFLYCCPRRGLL]
Crp4-2	cyclo[CYCRKGHCCKRGERVRGTCGIRFLYCCPRRGLL] ^c
Crp4-3	cyclo[CYCRKGHCCKRGERVRGTCGIRFLYCCPRRGLL] ^c
Crp4-R/A	<u>CYCRKGHCCKRGERVRGTCGIRFLYCCPRRGLL</u> ^d

^aThe first three amino acids of the Crp4 natural N-terminus are colored blue for reference. ^bAla residues replacing original Cys residues are colored magenta. ^cThe linkers added to assist in cyclization are colored green. ^dCys residues reduced and alkylated are underlined.

the newly formed loop. To facilitate the ligation, we used Cys^I (Crp4-1), Cys^{II} (Crp4-4), and Cys^V (Crp4-5) as the N-terminal residues in the corresponding linear precursors (Table 1). In addition, we also explored the effect of adding extra residues in the newly formed loop in the backbone-cyclized Crp4. Hence, we added an extra Gly residue (Crp4-2) and a Pro-Gly sequence (Crp4-3) between the native N- and C-terminal residues, Gly1 and Arg32, respectively (Table 1). These two additions should increase the flexibility of the new loop as well as facilitate the formation of the required turn.⁵⁶ In these two variants, we used only Cys^I as the N-terminal residue to allow for the cyclization reaction.

In Vitro Biosynthesis of Backbone-Cyclized Crp4 Peptides. All five linear Crp4–intein fusion precursors were expressed in BL21(DE3) cells at 30 °C and purified by affinity chromatography using chitin–Sephacrose beads. The Crp4–intein precursors have a chitin binding domain (CBD) fused at the C-terminus of the intein domain to facilitate purification. The expression yields of the different Crp4–intein constructs in the soluble cell lysate were estimated by UV spectroscopy and ranged from ≈5 mg/L for intein precursor Crp4-1 to ≈14 mg/L for precursor Crp4-5 (Table 2). The rest of the

Table 2. Expression Yields and Cleavage Percentages of Crp4–Intein Precursors in BL21(DE3) Cells and Yields of Cyclic and Folded Crp4 Variants after in Vitro Cleavage of the Intein

precursor	yield (mg/L)		% of soluble intein cleaved		yield ^c [µg/L (%) of cyclic/folded]
	S ^a	P ^b	in vivo	in vitro	
Crp4-1	5.5	142.1	23.9	84.2	200 (50%) ^d
Crp4-2	9.8	24.7	22.9	73.4	120 (20%) ^d
Crp4-3	10.5	94.3	20.7	70.4	174 (27%) ^d
Crp4-4	9.0	0	29.1	67.7	43 (10%) ^d
Crp4-5	13.9	0	7.5	45.6	35 (5%) ^d

^aSoluble cell lysate fraction. ^bInsoluble cell lysate fraction. ^cObtained after in vitro cleavage with GSH (see Experimental Procedures). ^dCyclization/folding yield based on the amount of precursor cleaved during the GSH-induced in vitro cleavage.

constructs gave similar expression yields in the soluble fraction (≈10 mg/L). All of the linear precursors using Cys^I as the N-terminal residue (Crp4-1, Crp4-2, and Crp4-3) also produced a

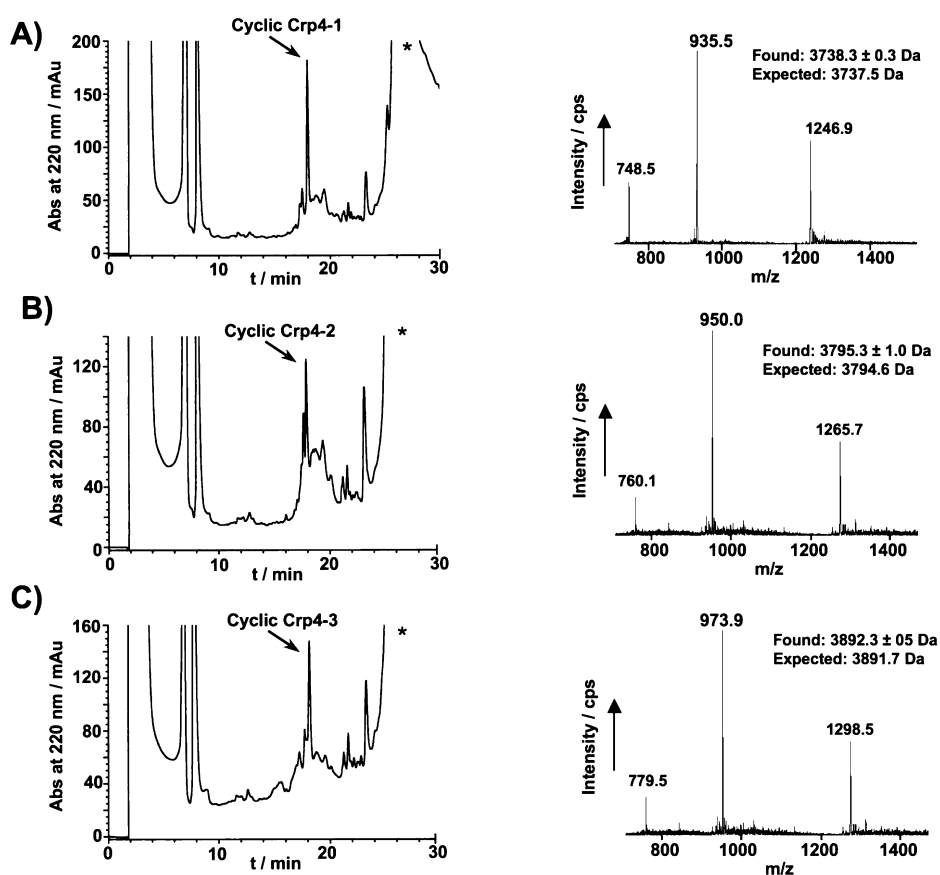


Figure 3. In vitro GSH-induced cyclization of precursors Crp4-1 (A), Crp4-2 (B), and Crp4-3 (C). On the left are the RP-HPLC chromatograms of the crude reaction and on the right the ES-MS data of the corresponding cyclic folded product indicated with an arrow. The large peak labeled with an asterisk is the intein–CBD protein. Experimental and theoretical molecular weights are shown with MS spectra.

significant amount of protein in the insoluble fraction ranging from 142 mg/L for Crp4-1 ($\approx 90\%$ of the total precursor expressed) to 24 mg/L for Crp4-2 ($\approx 70\%$ of the total precursor expressed). Interestingly, neither Crp4-4 nor Crp4-5 showed any significant expression in the insoluble fraction (Figure S1 of the Supporting Information), suggesting that the arrangement of the Crp4 peptide primary sequence plays an important role in the folding of the precursors and subsequent shuttling to inclusion bodies. Analysis of the purified fusion proteins by SDS–PAGE (Figure S1 of the Supporting Information) revealed that all of the linear precursors except Crp4-5 showed $\approx 20\%$ cleavage of the intein fusion in vivo when expressed in BL21(DE3) cells for 4 h at 30°C . In contrast, precursor Crp4-5 gave only 7% in vivo cleavage.

We next tested the ability of the different precursors to be cleaved in vitro using reduced glutathione (GSH). GSH has been shown to promote cyclization and concomitant folding when used in the biosynthesis of Cys-rich cyclic polypeptides.^{18,49,55} The cyclization/folding reaction was performed on the chitin beads where the corresponding precursors had been purified. The best cleavage/cyclization conditions were achieved using 100 mM GSH in phosphate buffer at pH 7.2 for 48 h. Under these conditions, $\approx 85\%$ of precursor Crp4-1 was cleaved in vitro (Table 2 and Figure S1 of the Supporting Information). Precursors Crp4-2 and Crp4-3 were also cleaved efficiently ($\approx 70\%$) under these conditions. In contrast, linear precursors Crp4-4 and Crp4-5 were cleaved less efficiently by GSH, $\approx 65\%$ for Crp4-4 and $\approx 45\%$ for Crp4-5. HPLC analysis of the crude cyclization mixture revealed in all the cases the

main peptide product was the corresponding folded backbone-cyclized Crp4 variant as revealed by ES-MS analysis (Figure 3 and Figure S2 of the Supporting Information). Other peptide peaks in the HPLC chromatograms were identified as incorrectly folded GSH adducts. Among the different linear precursors, Crp4-1 gave the best cyclization/folding yield (Figure 3A), producing $\sim 200\ \mu\text{g/L}$ cyclized Crp4-1 [$\approx 50\%$ of the theoretical yield (see Table 1)]. The cyclization/folding crude mixture for Crp4-2 and Crp4-3 (Figure 3B,C), both using the same ligation site as Crp4-1 but with different linker lengths for the new loop formed at the ligation site, gave lower yields (≈ 120 and $170\ \mu\text{g/L}$, respectively) than Crp4-1. The HPLC trace for the cyclization reaction of precursor Crp4-3 was, however, cleaner than that of Crp4-2, indicating the cyclization/folding was more efficient in that precursor (Figure 3B,C). The cyclization/folding yields for Crp4-4 and Crp4-5 (Figure S2 of the Supporting Information) were similar and estimated by HPLC to be ~ 40 and $\sim 35\ \mu\text{g/L}$, respectively (Table 2). These lower yields could be attributed, in part, to the less efficient cleavage of the intein in these precursors.

It is worth noting that during the cleavage of the intein precursors we found that the corresponding cyclized Crp4 peptides were able to bind strongly to the chitin beads under the conditions used for the GSH-induced cyclization/folding and required the use of 8 M GdmCl to elute the cyclized peptides from the solid support. This treatment also eluted the intein–CBD protein byproduct (Figure 3). Accordingly, we tested whether the cyclized Crp4 variants were binding to the chitin–Sephacrose beads or intein–CBD protein by incubation

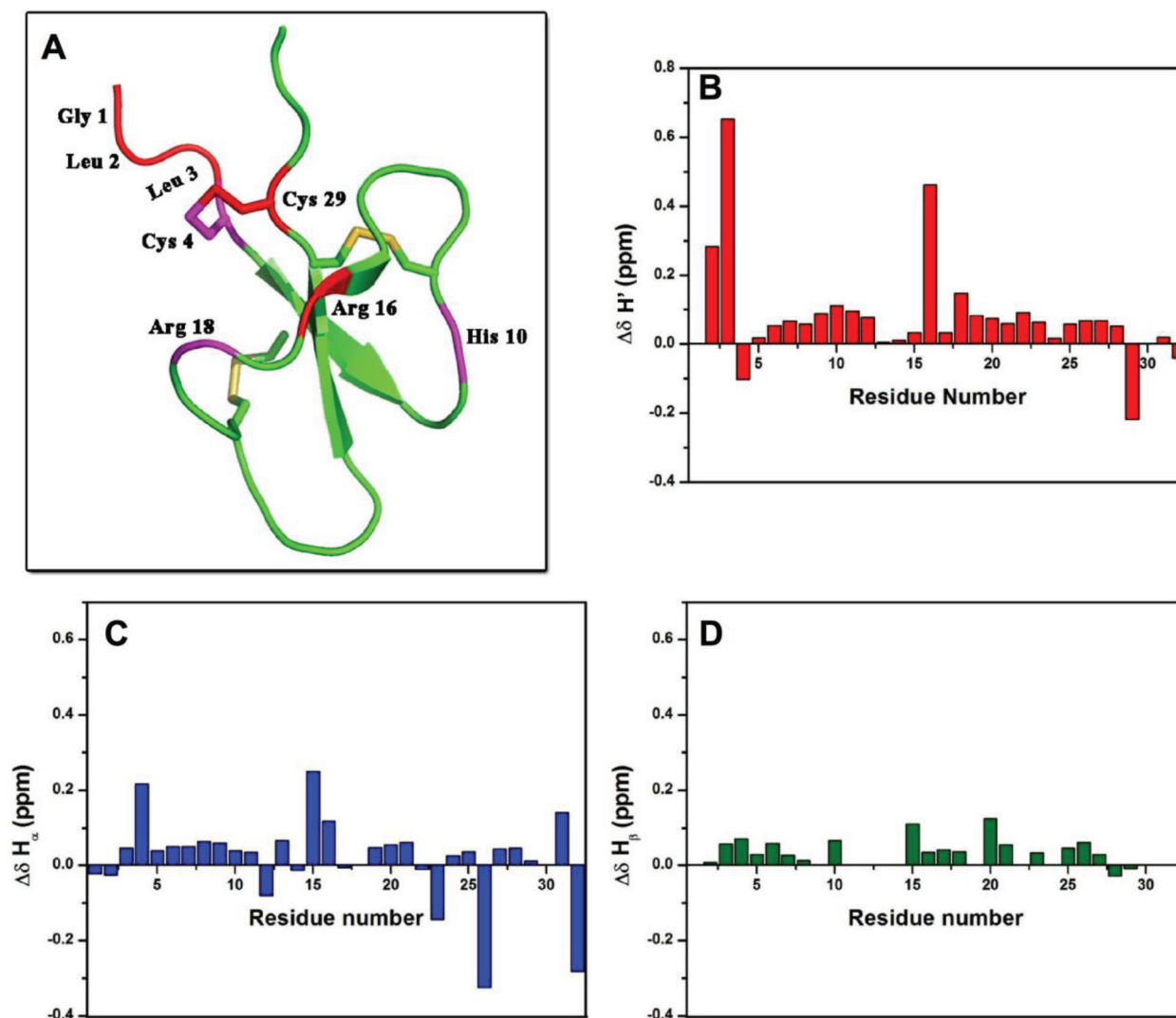


Figure 4. Chemical shifts differences of the backbone, H' and H^α , and side chain, H^β , protons between cyclic Crp4-1 and native Crp4-wt. (A) Ribbon diagram of native Crp4-wt showing the residues that exhibit a significant change in the chemical shift of the backbone amide after cyclization. Residues with a change in chemical shift larger than 0.2 ppm or between 0.1 and 0.2 ppm are colored red or magenta, respectively. Changes in the H' (B), H^α (C), and H^β (D) chemical shifts between native Crp4-wt and cyclic Crp4-1 defensins reflect minimal structural perturbations due to cyclization.

of purified cyclized Crp4-1 with either free, CBD bound, or intein–CBD-bound chitin–Sephadex beads. After being extensively washed, the beads in both cases were washed with 8 M GdmCl, and the amount of eluted Crp4-1 was quantified by HPLC. The results showed that approximately $\approx 25\%$ was able to bind free or CBD-bound chitin beads, and $\approx 90\%$ of cyclized Crp4-1 was bound to intein–CBD chitin beads (data not shown). This demonstrates that although cyclized Crp4 has some weak affinity for chitin–Sephadex beads, it was bound to the beads mainly by protein–protein interactions with the gyrase intein.

Structural Characterization of Cyclized Crp4 Defensins. Because the structure of native Crp4-wt has been elucidated by 1H NMR,³⁵ we used heteronuclear NMR spectroscopy to confirm that the biosynthetic cyclized Crp4 defensins were adopting a native α -defensin fold. Given that Crp4-wt and cyclized Crp4-1 have the same sequence, we first compared the assigned backbone amide and α (H' and H^α , respectively) proton chemical shifts of cyclized Crp4-1 with those published for the native Crp4-wt peptide (Table S2 of the

Supporting Information).³⁵ As shown in Figure 4, the chemical shift differences for most of the residues were smaller than 0.1 ppm, which indicates that cyclized Crp4-1 adopts a structure that is very similar to that of native Crp4-wt. We also saw a few residues that showed chemical shift differences of >0.1 ppm. Most of these residues, however, were located in the new loop formed close to the cyclization site, including residues Leu2, Leu3, Cys4, and Cys29 (Figure 4A). Interestingly, we also noticed a few residues (His10, Arg16, and Arg18) located away from the ligation site that presented relatively large chemical shift differences (Figure 4A). The chemical shift difference associated with H' of Arg16 was ≈ 0.4 ppm; meanwhile, for the other residues, the corresponding differences were relatively smaller (≈ 0.1 ppm). On the basis of these changes, it is very likely that the hydrogen bond between the carbonyl group of Cys29 and H' of Arg16 present in the structure of native Crp4-wt is broken in cyclized Crp4-1. This change would lead to changes in the backbone dihedral angles of the neighboring amino acids. This is in agreement with the fact that the differences observed for the chemical shifts of the side chain

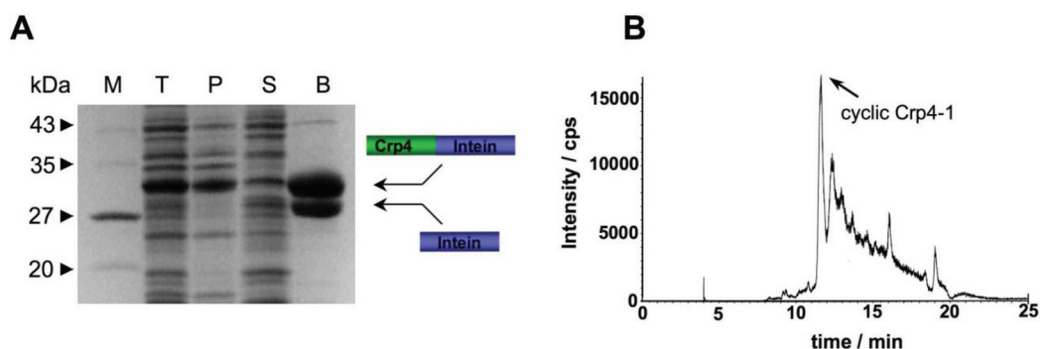


Figure 5. In vivo expression of cyclic Crp4-1 defensin in Origami2(DE3) cells. (A) SDS-PAGE analysis of the expression of Crp4-intein precursor Crp4-1. Lane M contained molecular mass protein markers, lane T total cell lysate, lane P insoluble cell lysate, lane S soluble cell lysate, and lane B affinity chromatography-purified Crp4-intein precursor. (B) Mass spectrum of the insoluble cell lysate following HPLC separation (see Experimental Procedures).

protons, H^β , are all smaller than 0.1 ppm (Figure 4D and Table S2 of the Supporting Information).

We also used heteronuclear $^1H\{^{15}N\}$ HSQC experiments to compare the structures of the different cyclized Crp4 variants. Uniformly ^{15}N -labeled cyclized Crp4 defensins were produced in vitro as described above, but *E. coli* was grown in minimal M9 medium containing $^{15}NH_4Cl$ as the only source of nitrogen. Recombinant expression of cyclized Crp4 defensins allows the introduction of NMR active isotopes (^{15}N and/or ^{13}C) in a very inexpensive fashion, thus facilitating the use of the SAR by NMR (structure-activity relationship by nuclear magnetic resonance)^{57,58} technique to study any molecular interaction between cyclized Crp4 defensins and their potential biomolecular targets. The HSQC spectra for all of the cyclized Crp4 variants were very well dispersed, indicating a well-folded structure (Figure S5 of the Supporting Information). As expected, the chemical shift differences for the backbone amide between the different cyclized Crp4 variants were also relatively small, the major differences being located in residues close to the ligation site and to Arg16 (Figure S5 of the Supporting Information). As expected, analysis of the HSQC spectrum of Crp4-2 revealed the presence of an additional Gly peak as compared to the spectrum of Crp4-1 (Figure S5B of the Supporting Information). In contrast, analysis of the HSQC spectrum of Crp4-3 revealed as many as nine additional peaks (Figure S5B of the Supporting Information). Careful integration of the intensities associated with the HSQC spectrum of cyclized Crp4-3 showed that 18 cross-peaks have intensities approximately 2 times lower than those of the rest of the peaks. These findings suggest that nine residues in cyclized Crp4-3 have two different conformations that could be exchanging at a very slow rate or not at all. We attributed the existence of two Crp4-3 conformations in solution to cis-trans isomerization of the proline in the new loop formed because most of the residues exhibiting amide peak doubling were located close to the newly formed loop.

In Vivo Biosynthesis of Cyclized Crp4 Defensins.

Encouraged by the results obtained with the in vitro GSH-induced cyclization/folding of the intein-Crp4 precursors, we decided to explore the expression of cyclized Crp4 inside *E. coli* cells. To accomplish this, we used intein precursor Crp4-1. This construct gave the best yield for the production of cyclized Crp4-1 in vitro (Table 2). The production of cyclized Crp4-1 was accomplished in Origami2(DE3) cells. These cells have mutations in the thioredoxin and glutathione reductase genes, which facilitates the formation of disulfide bonds in the

bacterial cytosol.⁵⁹ We have recently used these cells for the in vivo production of several disulfide-containing backbone-cyclized polypeptides.^{17,18,55}

The expression yield for the Crp4-1 precursor after overnight induction at room temperature with IPTG was approximately 5.5 mg/L. Under these conditions, ~35% of the Crp4-intein precursor was cleaved in vivo (Figure 5A). Using these numbers, we estimated the maximal amount of the cyclized Crp4-1 peptide that could be produced should be around 200 $\mu g/L$. Initial attempts to identify and quantify the amount of cyclized Crp4-1 in vivo by HPLC showed there to be very small amounts associated with the insoluble cell lysate and none detected in the soluble fraction. We were able to quantify the amount of cyclized Crp4-1 present in the insoluble cellular fraction using HPLC-MS/MS (Figure 5B). The yield was estimated to be $\approx 2 \mu g/L$, 100-fold lower than expected ($\approx 1\%$ yield). The cyclized Crp4-1 obtained in vivo had a mass corresponding to the folded product and coeluted with the purified product obtained in vitro, indicating that they were the same compound. The low efficiency observed for in vivo expression could be attributed to the toxicity of this defensin. α -Defensins are antimicrobial compounds that can bind and disturb the membranes of bacteria and have also been shown to inhibit the biosynthesis of peptidoglycan by binding to its precursor lipid II.^{60,61} It is likely that folded cyclized Crp4 may exert the same type of action when it is in the bacterial cytosol and could explain why Crp4-1 was found in the insoluble cell lysate. Moreover, we have shown that cyclized Crp4 defensins have affinity for the intein-CBD protein fusion, and therefore, any precursor protein present in the insoluble pellet could also bind cyclized Crp4 defensins, facilitating its immobilization to the insoluble cell lysate.

We also examined the expression of cyclized Crp4 defensins using the Crp4-4 and Crp4-5 precursors (Figure S1 of the Supporting Information), especially because the Crp4-5 precursor gave better in vivo cleavage ($\approx 70\%$). On the basis of the expression levels, the predicted yield for Crp4-4 is approximately 20 $\mu g/L$ and for Crp4-5 almost 200 $\mu g/L$. In both cases, the corresponding folded cyclized Crp4 variant was found in the insoluble cell lysate at yields similar to that found for Crp4-1 (1–2 $\mu g/L$). These three precursors gave very different yields for cyclization/folding in vitro. The fact that they provide a similar yield when expressed in vivo may suggest that the production in vivo could be limited by the cellular activity of the defensin.

Antimicrobial and Hemolytic Activities of Cyclized Crp4 Defensins. Native Crp4-wt has potent antimicrobial activities against a broad spectrum of microorganisms, including Gram-positive and Gram-negative bacteria.^{42,46,62} To explore the effect of cyclization on its biological activity, we tested the antimicrobial activities of the cyclized Crp4 defensins against several Gram-positive and Gram-negative bacteria. As shown in Figure 6, all cyclized defensins exhibited a dose-dependent

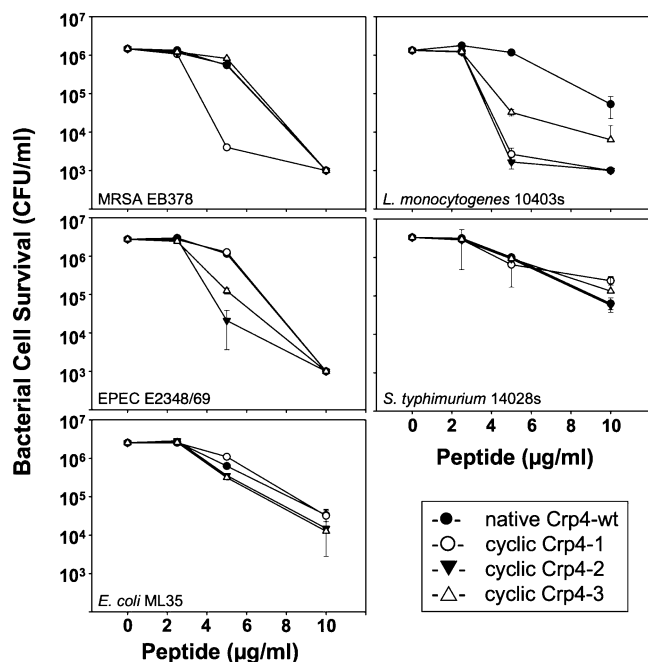


Figure 6. Bactericidal activities of Crp4-wt and cyclic Crp4 variants. Exponentially growing MRSA, *L. monocytogenes* 10403s, EPEC E2348/69, *S. typhimurium* 14028s, and *E. coli* ML35 were exposed to peptides at 37 °C in 50 μ L of PIPES-TSB buffer for 1 h (see Experimental Procedures). Following peptide exposure, the bacteria were plated on TSB-agar plates and incubated overnight at 37 °C. Surviving bacteria were counted as CFU at each peptide concentration, and count values below 1×10^3 CFU/mL signify that no colonies were detected.

killing of the bacteria tested, including methicillin resistant *Staphylococcus aureus* (MRSA) strain EB378 and enteropathogenic *E. coli* (EPEC) strain E2348/69. Overall, the cyclic peptides were most active against *Listeria monocytogenes* 10403s, killing 90–100% at 10 μ g/mL, and were least active against *Salmonella typhimurium* 14028s but still killed approximately 90% of the bacteria at 10 μ g/mL. The cyclic Crp4 peptides exhibited bactericidal activity equivalent to or in some cases greater than that of native Crp4-wt (Figure 6). For example, cyclized Crp4-1 and Crp4-2 were more potent than native Crp4-wt against *L. monocytogenes* 10403s at 5 and 10 μ g/mL. Similar results were obtained for cyclized Crp4-1 and Crp4-2 against MRSA and EPEC, respectively, at 5 mg/mL. These data demonstrate that cyclization preserves the biological activity of Crp4 and, for some bacterial cell targets, improves bactericidal activity.

Analysis of the antimicrobial activities in the presence of increasing NaCl concentrations showed minor differences at 160 mM NaCl, in which 10 μ g/mL native Crp4-wt killed 70% and cyclized Crp4-1 killed 80% of *L. monocytogenes* 10403s and *E. coli* ML35 (Figure S6a,b of the Supporting Information). These data contrast with previous studies of cyclic rabbit NP-1,

which was more active than natural NP-1 against *E. coli* in the presence of 100 mM NaCl.³⁷ This may be due to differences in the primary structures of Crp4 versus NP-1 (Figure 1), despite their highly similar three-dimensional folds. The antimicrobial activities of Crp4-wt and cyclic variants in the presence of 5% heat-inactivated human serum were attenuated, but 75–95% of EPEC exposed to 10 μ g/mL peptide were still killed (Figure S6C of the Supporting Information). These data suggest the cyclic Crp4 variants retain antimicrobial activity under conditions that mimic an in vivo environment and, therefore, have the potential to be developed as a drug lead, particularly against antibiotic resistant bacteria.

To assess the selectivity of Crp4 and cyclized Crp4 defensins, we tested the cytotoxicity of native Crp4-wt and cyclized Crp4-1 in a hemolysis assay against human RBCs. Both peptides lacked hemolytic activity at concentrations up to 100 μ g/mL, while the positive control peptide, melittin, gave approximately 75% hemolysis at the same concentration (Table 3). Additionally,

Table 3. Hemolytic Activities of Native and Cyclized Crp4 Defensins^a

peptide	0 μ g/mL	1 μ g/mL	12.5 μ g/mL	25 μ g/mL	50 μ g/mL	100 μ g/mL
Crp4-wt	4.2%	n/d ^b	4.1%	3.4%	3.0%	3.1%
Crp4-1	4.2%	n/d ^b	5.4%	5.8%	5.9%	4.9%
melittin	4.2%	3.6%	n/d ^b	n/d ^b	n/d ^b	74.6%

^aThe peptide melittin was used as a control. ^bNot determined.

native Crp4 has been found to be noncytotoxic to the mouse macrophage cell line, RAW 264.7 (unpublished work from the lab of A. J. Ouellette). These data demonstrate native and cyclic Crp4 are selective against bacteria.

Stability of Crp4 in Human Serum. Proteomics analyses of mouse colonic luminal contents have shown that intact, active α -defensins persist after secretion by Paneth cells of the small intestine.⁶³ Cryptdin peptides have been recovered from washing of mouse jejunum and ileum^{42,47} and the distal colonic lumen,⁶³ demonstrating their inherent resistance to proteolysis in the gastrointestinal environment conferred by the disulfide array. The stability of Crp4 was further assessed by incubation of native and cyclic peptides in 100% human serum, a location where Crp4 does not naturally occur (Figure 7). Both the native and cyclic peptides remained intact, with no hydrolysis of the N- and C-termini of native Crp4 (data not shown). In contrast, degradation of a disulfide-null mutant of native Crp4, in which the six Cys residues are mutated to Ala (Crp4-6C/A), and an S-carboxamidomethylated linear Crp4 (Crp4-R/A) (Table 1) began after just 2 min in human serum, and they were completely proteolyzed within 10–30 min (Figure 7, inset). Although Crp4-6C/A maintains potent antimicrobial activity in vitro, it is susceptible to degradation by matrix metalloproteinase (MMP) 7, the mouse pro- α -defensin convertase.^{50,64} These stability results open the possibility of developing Crp4 as a therapeutic agent. Although native Crp4 was stable in serum for 48 h, it is possible cyclized Crp4 variants may be more stable at longer time points or in vivo, when injected into or fed to animals.

DISCUSSION

Defensins are innate immune peptides that play an important role in the host defense of mammals. In this study, we have produced several backbone-cyclized variants of a mouse

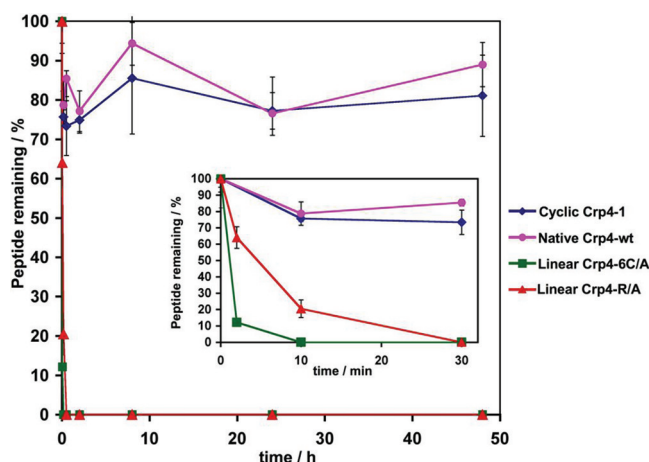


Figure 7. Stability of Crp4 and variants in human serum. Peptides at 100 $\mu\text{g/mL}$ were incubated with heat-inactivated serum at 37 $^{\circ}\text{C}$ and analyzed by C4 RP-HPLC. Linear Crp4-6C/A is a native Crp4-wt variant in which the six Cys are mutated to Ala. Linear Crp4-R/A corresponds to a linear Crp4-1 construct in which the six Cys residues are alkylated with iodoacetamide.

α -defensin in *E. coli* cells using modified protein splicing units. The cyclized peptides were characterized by NMR, antimicrobial activity, and serum stability.

The different cyclized versions of Crp4 were produced recombinantly either in vitro or in vivo. The best expression yield was obtained for Crp4-1 when it was cyclized in vitro ($\approx 200 \mu\text{g/L}$). The yields for the other cyclized Crp4 variants ranged from 170 to 35 $\mu\text{g/L}$ (Table 2). In vivo expression was by far less efficient with yields of $\approx 2 \mu\text{g/L}$. All of the examined cyclized Crp4 variants gave a similar yield when expressed in vivo that was independent of the precursor used. This strongly suggests that the amount of cyclized defensins that could be produced in vivo may be limited by the intracellular toxicity of the defensin. α -Defensins are antimicrobial compounds that can bind and disturb the membranes of bacteria and have also been shown to inhibit the biosynthesis of peptidoglycan by binding to its precursor lipid II.^{60,61} It is possible that folded cyclized Crp4 may exert the same type of action when it is in the bacterial cytosol, explaining the intracellular toxicity. Our data also indicate that cyclized Crp4 variants bind strongly to the intein–CBD fusion protein, specifically to the intein region. At this time, it is unknown how the peptide is binding to the protein. It may involve electrostatic interactions with the intein because Crp4 is highly cationic and the gyrase intein has an overall anionic charge. Interestingly, in vivo production of cyclized Crp4 defensins always provided the peptide on the insoluble fraction; therefore, it is possible that this occurs through binding to the insoluble intein–CBD protein. It may also be bound to insoluble bacterial membranes or cell wall precursors such as lipid II. Additional experiments to fractionate the pellet are necessary to delineate these results.

Structural characterization of the cyclized Crp4 variants by NMR revealed that they adopt structures that are very similar to that of native Crp4. This indicates that backbone cyclization did not significantly change the native fold of Crp4. The antimicrobial assays also demonstrated that backbone cyclization did not affect the biological activities of the peptide. In fact, some of the cyclized defensins exhibited better bactericidal activity (Figure 6). Interestingly, native and cyclic Crp4 showed no hemolytic activity against human red blood

cells, demonstrating their selectivity against bacteria. Additionally, Crp4-wt and cyclic Crp4-1 defensins both demonstrated high stability to human serum, with half-lives of >48 h. In contrast, mutation or alkylation of the Cys residues resulted in complete degradation of the corresponding linear peptides by human serum in <30 min. On the basis of these data, it is likely the three disulfide bonds are essential components in the stabilization of the peptide structures. The presence of the disulfide array has previously been shown to be a necessary component in resistance to proteolytic degradation but does not affect antimicrobial activity.⁵⁰ Altogether, these properties make cyclized Crp4 defensins promising scaffolds for drug development of novel antibiotics, although further studies may be required to evaluate their metabolic stability and bioavailability.

Novel antimicrobial agents are necessary to overcome the threat of prevalent antibiotic resistant pathogens. On the basis of the data reported here, this peptide can potentially be used as a stable scaffold to generate more enhanced antimicrobial drugs. Although a library of Crp4 sequences is not useful in bacterial expression systems, they have the potential to be expressed in yeast or mammalian cells providing there is no intracellular cytotoxicity. This may be useful for in vivo screening against intracellular pathogens, including viruses and parasites.⁶⁵ Studies have shown that antimicrobial peptides can decrease the viability of intracellular *Mycobacterium tuberculosis*⁶⁶ and inhibit the proliferation of intracellular *L. monocytogenes* in macrophages;⁶⁷ therefore, the expression of defensin libraries in mammalian cells is a promising method for developing and screening for more effective antimicrobials against intracellular pathogens.

■ ASSOCIATED CONTENT

● Supporting Information

Supplementary results mentioned in the text, including two tables and seven figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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■ ABBREVIATIONS

CBD, chitin binding domain; CFU, colony-forming unit; Crp4, cryptdin 4; DPBS, Dulbecco's phosphate-buffered saline;

EDTA, ethylenediaminetetraacetic acid; EPEC, enteropathogenic *E. coli*; GdmCl, guanidinium chloride; GSH, reduced glutathione; HPLC, high-performance liquid chromatography; HSQC, heteronuclear single-quantum coherence; IPTG, isopropyl β -D-1-thiogalactopyranoside; NCL, native chemical ligation; NMR, nuclear magnetic resonance; MRSA, methicillin resistant *St. aureus*; NOESY, nuclear Overhauser effect spectroscopy; PIPES, 1,4-piperazinediethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; RBCs, red blood cells; SFTI-1, sunflower trypsin inhibitor 1; TFA, trifluoroacetic acid; TOCSY, total correlation spectroscopy; TSP, trypticase soy broth; UV, ultraviolet.

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