The Single Disulfide-Directed β-Hairpin Fold: Role of Disulfide Bond in Folding and Effect of an Additional Disulfide Bond on Stability

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Disulfide bonds play a key role in the oxidative folding, conformational stability, and functional activity of many peptides. A few disulfide-rich peptides with privileged architecture such as the inhibitor cystine knot motif have garnered attention as templates in drug design. The single disulfide-directed β-hairpin (SDH), a novel fold identified more recently in contryphan-Vc1, has been shown to possess remarkable thermal, conformational, and chemical stability and can accept a short bioactive epitope without compromising the core structure of the peptide. In this study, we demonstrated that the single disulfide bond is critical in maintaining the native fold by replacing both cysteine residues with serine. We also designed an analogue with an additional, non-native disulfide bridge by replacing Gln1 and Tyr9 with Cys. Contryphan-Vc11–22[Q1C, Y9C] was synthesised utilising orthogonal cysteine protection and its solution structure determined using solution NMR spectroscopy. This analogue maintained the overall fold of native contryphan-Vc1. Previous studies had shown that the β-hairpin core of contryphan-Vc1 was resistant to proteolysis by trypsin and α-chymotrypsin but susceptible to cleavage by pepsin. Contryphan-Vc11–22[Q1C, Y9C] proved to be completely resistant to pepsin, thus confirming our design strategy. These results highlight the role of the disulfide bond in maintaining the SDH fold and provide a basis for the design of more stable analogues for peptide epitope grafting.

Introduction

Disulfide bonds are common structural motifs in bioactive peptides and proteins, including hormones, neurotransmitters, growth factors, enzyme inhibitors, and antimicrobial peptides.[1] Disulfide bond formation is critical for the proper folding of many bioactive peptides and proteins, and it is generally accepted that disulfide bonds enhance their thermodynamic stability.[2] Disulfide-rich peptides such as those incorporating the inhibitor cystine knot (ICK) motif[3] and cyclotides[4] are commonly used as molecular scaffolds for peptide grafting owing to their high stability and bioavailability.[5]

Recently, contryphan-Vc1, a peptide identified in proteomic studies on the venom gland of an Australian cone snail Conus victoriae,[6] was shown to have a unique peptide fold designated the single disulfide-directed β-hairpin (SDH).[7] The fold of this single disulfide-containing peptide closely resembled that of the multiple-disulfide-containing ICK structural motif.[3] Intriguingly, the SDH fold was found to possess remarkable thermal, conformational, and chemical stability and was able to accept a short bioactive epitope as an insert without compromising the core structure of the peptide.[8] An analogue, sCon-Vc11–22[NNN12–14], was engineered to incorporate the NNN sequence from inducible nitric oxide synthase (NOS), which mediates its binding to the SPRY domain and SOCS-box containing (SPSPB) proteins.[9] Apart from maintaining the SDH fold of the peptide, sCon-Vc11–22[NNN12–14] also bound to hSPSB2, albeit with 1.3 μM affinity, which is 50-fold higher than the NNN motif,[8] suggesting the potential value of utilising the SDH fold as a scaffold for the presentation of peptide epitopes.

Even though the SDH fold of contryphan-Vc1 exhibited remarkable thermal, chemical, and conformational stability, the peptide had only limited proteolytic stability.[8] The β-hairpin core of contryphan-Vc1 was resistant to cleavage by the enzymes trypsin and α-chymotrypsin but susceptible to cleavage by pepsin.[9] Pepsin is an aspartic protease that
preferentially cleaves at Phe, Tyr, Trp, and Leu in position P1 or P1'.[10] Selective residue replacement to make the peptide resistant to digestion by a particular protease is one of the methods to overcome the susceptibility of a peptide towards a protease. However, the sequence of the truncated contryphan-Vc1, Con-Vc11–22, contains five sites that are potentially susceptible to cleavage by pepsin (Trp2, Tyr7, Tyr9, Leu13 and 20), and although these sites are unlikely to be equally susceptible to proteolysis, other approaches have been considered.

Introducing disulfide bonds is a common approach to increasing the stability of peptides and proteins in biomedical and industrial applications, and various proteins have been successfully engineered for increasing thermal stability in this way.[11] However, not all engineered disulfides afford an increase in stability, and there are several reports of destabilising disulfides.[12] Disulfide bonds are believed to decrease the conformational entropy and raise the free energy of the denatured state, thus increasing the relative stability of the folded protein conformation.[13] Given that native disulfide bonds provide considerable stability to proteins,[14] it follows that the addition of a new disulfide crosslink to a peptide or protein might increase its stability.[15] An engineered disulfide bridge in subtilisin E was shown to increase thermal stability considerably.[16] Proteases such as thermolysin have been shown to gain stability by introduction of an additional disulfide bridge.[17] Introducing disulfide bonds can, apart from increasing the thermodynamic and kinetic stability of a protein, also enhance the proteolytic stability of a peptide or protein.[17,18]

In order to enhance the proteolytic stability of contryphan-Vc1 and overcome the limitation of accepting only short epitopes, we introduced an additional disulfide bond in an effort to further stabilise the SDH fold. The residues Gln1 and Tyr9 were chosen for replacement by Cys based on their proximity in the structure of contryphan-Vc1[17] and inspection of peptide sequence similarities. The solution structure of contryphan-Vc11–22[Q1C, Y9C] was determined using NMR spectroscopy and its proteolytic stability was assessed in the presence of trypsin, z-chymotrypsin, and pepsin. We also probed the role of the native disulfide bond of contryphan-Vc1 in maintaining the SDH fold by replacing the Cys3 and Cys16 residues with Ser.

**Experimental**

**Peptide Synthesis**

Contryphan-Vc11–22[Q1C, Y9C] and contryphan-Vc11–22[C3S, C16S] were synthesised on a PTI Instruments PS3 peptide synthesiser, using Rink amide 4-[(2,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxacyetamido-aminomethyl] (AM) resin and standard Fmoc-protected amino acids (Fmoc = fluorenylmethyloxycarbonyl).[19] Contryphan-Vc11–22[Q1C, Y9C] disulfides were synthesised using orthogonally protected cysteine residues to facilitate controlled disulfide bond oxidation. Cys1 and Cys9 were protected using S-acetamido methyl protective groups while Cys3 and Cys16 were protected using S-trityl groups. Cleavage from the resin was performed over 2 h with a mixture of 3,6-dioxo-1,8-octanedithiol, trisopropylsilane, 1,3-dimethoxybenzene, and trifluoroacetic acid (DODT/TIPS/DMB/TFA at ratio of 2:5 : 2.5 : 5 : 92.5 by volume). The cleavage mixture was purged with nitrogen and the crude peptide was precipitated with ice-cold diethyl ether, washed three times with the same solvent, and dried under vacuum. The peptide was subsequently folded and partially oxidised in 0.1 M ammonium bicarbonate at a peptide concentration of 0.3 mg mL−1 and pH 8.0. The partially folded peptide where the disulfide bond between Cys3 and 16 formed by air oxidation was purified by reversed-phase HPLC (RP-HPLC) on a Phenomenex® Luna C18 column (100 Å, 5 μm, 10 × 10 mm) using a gradient of 5–95% B (A: 99.9% H2O, 0.1% TFA; B: 80% acetonitrile (ACN), 19.9% H2O, 0.1% TFA) over 30–60 min and the sample was lyophilised. The second disulfide bond, between Cys1 and 9, was formed by iodine oxidation, where 0.5 mM of lyophilised partially folded peptide was incubated for 30 min in the presence of 5 mM iodine dissolved in 50% ACN. The reaction was stopped using 100 mM sodium ascorbate solution and the fully oxidised peptide was purified by RP-HPLC and checked by liquid chromatography–mass spectrometry (LC-MS). Similarly, contryphan-Vc11–22 [C3S, C16S] was synthesised on a PTI Instruments PS3 peptide synthesiser using Rink amide AM resin as described previously.[19] The sequences of the peptides used in this study are shown in Table 1.

**NMR Spectroscopy**

All NMR spectra were acquired on a Bruker 600 MHz spectrometer equipped with a cryogenically cooled triple-resonance probe. Lyophilised peptide was dissolved in either 93% H2O/7% D2O or 100% D2O (pH 4.0). One-dimensional (1D) 1H NMR spectra were acquired at different temperatures between 5 and 40°C, at intervals of 5°C, and between pH 3 and 9. All two-dimensional (2D) NMR spectra (TOCSY, NOESY, 13C-HSQC, and 15N-HSQC; TOCSY = total correlation spectroscopy; HSQC = heteronuclear single quantum coherence) utilised for sequence-specific assignments and structure calculations were acquired at pH 4 and 40°C. 2D homonuclear TOCSY spectra with a spin lock time of 80 ms were acquired using the DIPSI-2 pulse sequence with excitation sculpting for water suppression at 15, 20, 25, and 40°C. 2D NOESY spectra were acquired with mixing times of 50, 200, and 300 ms to analyse the time-dependence of NOE intensities. 13C-HSQC and 15N-HSQC spectra were acquired for carbon and nitrogen chemical shifts respectively. For determination of potential backbone amide hydrogen bonds in contryphan-Vc11–22[Q1C, Y9C], a series of 1D 1H spectra was acquired at 10, 15, 20, 25, and 30°C and the amide resonance temperature coefficients were determined from the slopes of linear least-squares fits to the data. A sine-bell squared window function was used for processing spectra. All spectra were processed using Bruker TopSpin (version 3.2) and analysed using CcpNmr Analysis (version 2.1.5).[20]

**Structure Calculation**

The intensities of cross-peaks in the NOESY spectra with a mixing time of 300 ms were utilised to generate distance

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Contryphan-Vc11–22</td>
<td>QWCGPGYAYNPVLGICTTLSR</td>
</tr>
<tr>
<td>Contryphan-Vc11–22[C3S, C16S]</td>
<td>QWSQPGYAYNPVLGICTTLSR</td>
</tr>
<tr>
<td>Contryphan-Vc11–22[Q1C, Y9C]</td>
<td>CWCGPGYACNPVLGICTTLSR</td>
</tr>
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*Both the peptides are C-terminally amidated.*
constraints. $^3$JHN-Ha coupling constants were measured from 1D $^1$H spectra, which yielded seven $\phi$ angle constraints. These $\phi$ angles were restrained to $-120 \pm 30^\circ$ for $^3$JHN-Ha $\geq$ 8.0 Hz and $-65 \pm 25^\circ$ for $^3$JHN-Ha $\leq$ 6.0 Hz. Three distance constraints were added for the disulfide bridge as follows: 2.00, 3.00, and 3.00 Å for S(i)–S(j), S(i)–Cβ(j), and S(j)–Cβ(i) respectively.

The structures of contryphan-Vc11–22[Q1C, Y9C] were calculated using CYANA (version 3.0). Structure calculations were performed using 213 inter-proton distance constraints derived from the NOESY spectrum, seven dihedral angle restraints derived from $^3$JHN-Ha coupling constants measured from 1D $^1$H NMR spectra, and three disulfide bond restraints. The root-mean-square deviation (RMSD) values for these structures were assessed using MolMol (version 2K.1). Structural figures were prepared using PyMOL (version 1.5.0.4).

**Molecular Dynamics (MD) Simulations**

MD simulations were carried out using GROMACS version 5.0.4 with the GROMOS 54a7 united-atom force field and a 2 fs time step. Temperature coupling made use of the velocity rescale algorithm with a reference temperature of 293 K. Pressure coupling used the Parinello–Rahman algorithm, with reference pressure of 1 bar and compressibility of $4.5 \times 10^{-5}$ bar$^{-1}$ (1 bar = 1 x 10$^5$ Pa). Initial structures were generated using the Maestro software package (v. 10.3.014) by in silico mutation of the published structure of recombinant contryphan-Vc11–22[Z1Q] (Protein Data Bank (PDB) code: 5KKM). These structures were solvated with simple point-charge (SPC) water and subjected to a steepest-descent minimisation of 2000 steps to remove bad van der Waals contacts between atoms. Temperature equilibration (without pressure coupling) was run for 10000 steps. Isotropic pressure coupling was then applied for 500000 steps. Following equilibration, the simulation production runs were executed for 300 ns each. The resultant trajectories were visualised with the Visual Molecular Dynamics (VMD) software package (v. 1.9.2) and analysed with VMD and GROMACS built-in tools. The RMSD and radius of gyration ($R_g$) were calculated based on data extracted using the rms and gyrate commands respectively of GROMACS and plotted using the gnuplot software package (v. 5.0).

**Proteolysis Assays**

Proteolysis assays were performed at a 250 : 1 substrate (peptide)/enzyme ratio with pepsin, trypsin, and $\alpha$-chymotrypsin. Digestion...
assay products were analysed by LC-MS (0–60 % ACN gradient, 10 min). Trypsin (EC 3.4.21.4, Sigma) and α-chymotrypsin (EC 3.4.21.1, Sigma) stocks were prepared in 50 mM Tris, 100 mM NaCl (pH 7.4), and pepsin (EC 3.4.23.1, Sigma) stocks were prepared in 10 mM HCl (pH 2). The reactions for trypsin and α-chymotrypsin were performed in 50 mM Tris, 100 mM NaCl, 2 mM CaCl₂, pH 7.4. The reaction for pepsin was performed in 1 mM HCl, pH 2. The trypsin and α-chymotrypsin reactions were quenched with 0.1 % TFA, and the pepsin reaction with 50 % 1 M NaOH. For all assays, peptides were incubated with enzyme at 37°C for up to 4 h. Full-length contryphan-Vc1 was used as control substrate.

Results

The Native Disulfide Bond is Critical for Maintenance of the SDH Fold

We have previously produced recombinant full-length contryphan-Vc1, in which the native N-terminal pyroGlu was replaced by Gln (rCon-Vc1[Z1Q]), as well as a truncated analogue (rCon-Vc11–22[Z1Q]). Backbone 15N relaxation measurements for rCon-Vc1[Z1Q] showed that the N-terminal structured domain of the peptide is ordered up to Thr19, whereas the C-terminal region (amino acid residues 20–31) is flexible. The solution structure of truncated rCon-Vc11–22[Z1Q] was similar to that of the full-length peptide, indicating that the flexible C-terminus did not affect the structured domain. All peptides investigated in the present study were synthetic, but with an N-terminal Gln rather than pyroGlu because the Gln and pyroGlu analogues had the same structures.

Contryphan-Vc11–22[C3S, C16S], an analogue of Con-Vc11–22[Z1Q] in which both Cys3 and Cys16 were replaced with Ser, was synthesised to probe the importance of the native disulfide bond in the folding and maintenance of the SDH fold of the contryphan-Vc1. 1D 1H NMR spectra of Con-Vc11–22[C3S, C16S] recorded at 20°C and pH 4.0 showed that the peptide was not well folded, as the peak dispersion was lost when compared with the native contryphan-Vc1 (Fig. 1a, b). Both amide and Hα secondary chemical shift plots confirmed that contryphan-Vc11–22[C3S, C16S] had not formed the SDH fold (Fig. 1d), so a structure calculation was not attempted.

Analysis of the MD Simulation Trajectories of Contryphan-Vc11–22[Q1C, Y9C]

In order to assess whether introduction of an additional disulfide bond between residues 1 and 9 of the contryphan-Vc1 was compatible with the native structure, we performed MD simulations on contryphan-Vc11–22[Q1C, Y9C] and calculated global RMSD and radius of gyration across the 300-ns simulation time of each trajectory. RMSD values indicated the extent to which the structure was changing, while the radius of gyration allowed the compactness of the structure to be monitored. The RMSD plot of the simulation trajectory over 300 ns indicated that the molecule was stable throughout the simulation following initial changes during the first 50 ns (Fig. 2a). The radius of gyration was generally constant throughout the simulation time course, again implying that the structure of the molecule was stable and the peptide did not unfold (Fig. 2b). The RMSD and radius of gyration over the 200 ns simulation time of each trajectory for full-length contryphan-Vc1 between residues Cys3 to Cys16 were calculated as a control (Fig. 2c, d).

Synthesis and Purification of Contryphan-Vc11–22[Q1C, Y9C]

Contryphan-Vc11–22[Q1C, Y9C] was synthesised using orthogonal cysteine protection. Cys1 and Cys9 were protected using S-acetamido methyl protective groups and Cys3 and
Contryphan-Vc11–22[Q1C, Y9C] Sequence-Specific Resonance Assignments for a identified by the observation of strong NOE Gln4 tary Material). The spin systems for Pro5 and Pro11 were spin systems and sequential assignments (Fig. S1, Supplementary served as good starting points for the simple identification of such as Ala8, Thr17 and 19, Leu13 and 20, and Ile15 and 18 NOESY spectra. Amino acid residues containing methyl groups resonance assignments were made utilising 2D TOCSY and 2D mixing time) spectra acquired at 30°C. Sequential 1D 1H NMR spectra of contryphan-Vc11–22[Q1C, Y9C] showed of double-quantum filtered-correlation spectroscopy (DQF-COSY), TOCSY (80 ms spin-lock time) and NOESY (300 ms mixing time) spectra acquired at 30°C and pH 4.0. Sequential resonance assignments were made utilising 2D TOCSY and 2D NOESY spectra. Amino acid residues containing methyl groups such as Ala8, Thr17 and 19, Leu13 and 20, and Ile15 and 18 served as good starting points for the simple identification of spin systems and sequential assignments (Fig. S1, Supplementary Material). The spin systems for Pro5 and Pro11 were identified by the observation of strong NOE Gln4–Pro5 and Asn10–Pro11 cross peaks, which confirmed the trans conformations of both X–Pro bonds (Fig. S1B, Supplementary Material). Complete backbone and side chain 1H and 15N resonance assignments were made for all spin systems (Fig. S1C and Table S1, Supplementary Material).

**Solution Structure of Contryphan-Vc11–22[Q1C, Y9C]**

In order to investigate the influence of the additional disulfide bond between residues 1 and 9 on contryphan-Vc1, the structure of contryphan-Vc11–22[Q1C, Y9C] was determined and compared with that of contryphan-Vc11–22[Z1Q]. Contryphan-Vc11–22[Q1C, Y9C] retained the fold of contryphan-Vc1 even after the introduction of an additional disulfide bond and its structure is almost identical to that of contryphan-Vc11–22[Z1Q] (Fig. 5b). Superimposition of the structures with the lowest target function of contryphan-Vc11–22[Q1C, Y9C] and contryphan-Vc11–22[Z1Q] yielded an RMSD of 0.9 Å over Cα atoms of residues 3–16 (Fig. 5b). The four residues Glu4, Pro5, Gly6, and Tyr7 form a type-II β-turn that leads to the first β-strand. Ala8, Cys9, and Asn10 form the first β-strand and Ile15, Cys16, and Thr17 make up the other strand. Pro11, Val12, Leu13, and Gly14 form a β-hairpin. Similarly to contryphan-Vc11–22[Z1Q], residues after the second β-strand, Ile18–Arg22, are unstructured. The amides of Ala8, Asn10, Thr17, and Ile15 had temperature coefficients less negative than −4.75 ppb K−1, indicating that these residues participate in hydrogen bonding. The hydrogen bonds inferred from the structure are between Ala8 HN and Thr17 O, Asn10 HN and Ile15 O, Ile 15 HN and Asn10 O, and Thr17 HN and Ala8 O, all of which are part of the anti-parallel β-sheet and identical to the hydrogen bonds observed in contryphan-Vc11–22[Z1Q]. Structural constraints are summarised in Table 2.

**Proteolytic Stability of Contryphan-Vc11–22[Q1C, Y9C]**

The proteolytic stability of contryphan-Vc11–22[Q1C, Y9C] was assessed utilising the enzymes trypsin, α-chymotrypsin, and pepsin. Contryphan-Vc11–22[Q1C, Y9C] was fully resistant to
trypsin, as expected because this peptide has no tryptic cleavage site (Fig. 6a), in contrast to full-length contryphan-Vc1, which is cleaved following Arg22. In the presence of a-chymotrypsin, contryphan-Vc11–22[Q1C, Y9C] showed a major peak in the LC-MS chromatogram, with mass 2153.5 Da, corresponding to the truncated product contryphan-Vc11–20[Q1C, Y9C] (Fig. 6b). Contryphan-Vc11–22[Q1C, Y9C] did not show any cleavage peaks in the presence of pepsin despite having multiple cleavage sites for that enzyme, indicating that the peptide was now resistant to pepsin (Fig. 6c). In contrast, the native peptide was fully digested under the same conditions (Fig. S2, Supplementary Material).

Fig. 4. (a) 1D 1H NMR spectra of contryphan-Vc11–22[Q1C, Y9C] recorded at different temperatures ranging from 278 to 313 K at pH 4.0. (b) Comparison of 1D 1H NMR spectra of contryphan-Vc11–22[Q1C, Y9C] (red) with con-Vc11–22[Z1Q] (blue). (c) 1D 1H NMR spectra of contryphan-Vc11–22[Q1C, Y9C] over the pH range 2–7 at 303 K in water containing 7% 2H2O.
Distance constraints

Average pairwise RMSD [Å]
Backbone [Å] (N, C\(_\alpha\), C) 0.27 ± 0.06
All heavy atoms [Å] 0.55 ± 0.07

Ramachandran analysis
Residues in most favoured regions [%] 68.1
Residues in additionally allowed regions [%] 31.9
Residues in generously allowed regions [%] 0.0
Residues in disallowed regions [%] 0.0

Discussion

In the present study, we have demonstrated that the native disulfide bond is critical for maintenance of the SDH fold and that an additional disulfide bond can be introduced in contryphan-Vc1–22 by replacing Gln1 and Tyr9 with Cys without disrupting this fold. Temperature-dependent conformational averaging and partial self-association were observed for contryphan-Vc1–22[Q1C, Y9C], with the line widths becoming sharper and the spectral dispersion improving above 25°C. Introduction of this additional disulfide bond also enhanced proteolytic stability, with contryphan-Vc1–22[Q1C, Y9C] being completely resistant to pepsin digestion, in contrast to native contryphan-Vc1, which was highly susceptible to pepsin.

The SDH fold is a unique fold where the disulfide linkage is not the usual direct cross-strand favoured for β-hairpin formation. Previously, we have shown that the disulfide bond is necessary to maintain the stability of the SDH fold by reducing the disulfide bond with an excess of Tris(2-carboxyethyl)-phosphine (TCEP).[8] In this study, the NMR spectral changes in the disulfide-deficient contryphan-Vc1 analogue Contryphan-Vc1–22[C3S, C16S] demonstrated that the disulfide bond is critical not only in the maintenance of the SDH fold but also for folding of the peptide.

One-dimensional \(^1\)H NMR spectra of contryphan-Vc1–22[Q1C, Y9C] recorded at 5–25°C and pH 4.0 showed that most of the peaks were broad, indicating the presence of different conformations in exchange and/or partial self-association. As the temperature was increased beyond 25°C, the peaks sharpened and the dispersion became similar to that of native contryphan-Vc1, suggesting that the folded conformation was being stabilised at higher temperatures (Fig. 4). This phenomenon of inverse temperature transition[25] is commonly seen in the case of elastin and elastin-like peptides, which transition from a disordered (extended) to an ordered (folded) conformation on heating. The transition is also influenced by pH and the molecular basis is attributed to interaction of the elastin molecules with solvent water and the collapse of the side-chain atom hydrophobic interactions.[26]

The cause of peak broadening at low temperature in the case of contryphan-Vc1–22[Q1C, Y9C] is not yet clear, and further study is warranted to understand the molecular basis. MD simulations performed at 20°C showed that the RMSD and radius of gyration were stable, with no evidence of significant conformational averaging. Increased hydrophobicity of the peptide associated with introduction of the additional disulfide bond might account for partial aggregation, and the reversal of such aggregation at higher temperature suggests that the peptide needs additional energy to drive hydrophobic collapse and folding. 1D \(^1\)H NMR spectra of contryphan-Vc1–22[Q1C, Y9C] showed subtle chemical shift changes in the pH titration between pH 2 and 7, but the overall spectral dispersion was maintained, indicating the stability of the peptide over this pH range.

The solution structure of contryphan-Vc1–22[Q1C, Y9C] was similar to that of native contryphan-Vc1, with superposition of the structures of contryphan-Vc1–22[Q1C, Y9C] and con-Vc1–22[Z1Q] with the lowest target function showing an RMSD of 0.9 Å over C\(_\alpha\) atoms of residues 3–16 (Fig. 5). Thus, contryphan-Vc1–22[Q1C, Y9C] maintained the overall fold of native contryphan-Vc1, supporting the validity of our design based on the structures of other ICK peptides and the proximity and orientation of the side chains of Gln1 and Tyr9 in the structure of the native peptide.[7,8] Maintenance of the native fold of the contryphan-Vc1 after addition of an extra disulfide linkage also emphasises the similarities between the ICK and SDH folds.

An important driver for the introduction of an additional disulfide was to enhance the proteolytic stability of contryphan-Vc1. Contryphan-Vc1–22[Z1Q] was resistant to trypsin.
In contrast, contryphan-Vc1 1–22[Q1C, Y9C] was resistant to 313 K (Table S1), overlay of two-dimensional 1H NMR spectra for contryphan-Vc1 1-22[Q1C, Y9C]) at pH 4.0, showing Gln4 - Pro5 and Asn10 - Pro11 cross peaks, suggesting that both prolines are in the trans conformation, 15N-HSQC spectrum of 1 mM contryphan-Vc1 1-22[Q1C, Y9C] at pH 4.0 and 40°C in water containing 7% 2H2O (Fig. S1), reversed-phase HPLC analyses of rCon-Vc1 1-22[Z1Q] treated with trypsin, pepsin and chymotrypsin, similarly to con-Vc11–22[Z1Q].

The Single Disulfide-Directed engineering is successful in overcoming the limitation of contryphan-Vc1. Further studies to assess whether contryphan-Vc1 will be a cleavage site for the enzyme, and hence that mutating Tyr9 to Cys9 contributed to the resistance to pepsin digestion. Enhancing the proteolytic stability of native contryphan-Vc1 by constraining the peptide with an additional disulfide linkage demonstrates a well-utilised concept in which the addition of a disulfide linkage stabilises polypeptides susceptible to proteolytic degradation.[13] Despite the observed conformational averaging and self-association at lower temperatures, the peptide is still an attractive candidate for use as a scaffold owing to its conformational stability at higher temperature, maintenance of the overall SDH fold, relative ease of production compared with other peptides having more disulfide bonds, and proteolytic stability.

In summary, we have shown that engineering contryphan-Vc1 with an additional disulfide bond maintained the SDH fold and enhanced the proteolytic stability of native contryphan-Vc1. Further studies to assess whether contryphan-Vc1 1-22[Q1C, Y9C] can accept epitopes larger than three residues, for example the full DINNN epitope that mediates binding to the SPBS proteins, will establish whether disulfide engineering is successful in overcoming the limitation of accepting short epitopes (~three residues) by contryphan-Vc1 1-22[Z1Q].[8]

**Supplementary Material**

Chemical shifts for contryphan-Vc1 1-22[Q1C, Y9C] at pH 4.0, 313 K (Table S1), overlay of two-dimensional 1H NMR spectra TOCSY and NOESY of contryphan-Vc1 1-22[Q1C, Y9C] recorded at 40°C, region of two-dimensional NOESY spectrum of 1 mM contryphan-Vc1 1-22[Q1C, Y9C] at pH 4.0 and 40°C in water containing 7% 2H2O (Fig. S1), reversed-phase HPLC analyses of rCon-Vc1 1-22[Z1Q] treated with trypsin, pepsin and chymotrypsin (Fig. S2) are available on the Journal’s website.

**Conflicts of Interest**

The authors declare no conflicts of interest.

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