Protein Engineering

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Diaminodiacid Bridges to Improve Folding and Tune the Bioactivity of Disulfide-Rich Peptides**

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Abstract: *Disulfide-rich peptides containing three or more disulfide bonds are promising therapeutic and diagnostic agents, but their preparation is often limited by the tedious and low-yielding folding process. We found that a single cystine-to-diaminodiacid replacement could significantly increase the folding efficiency of disulfide-rich peptides and thus improve their production yields. The practicality of this strategy was demonstrated by the synthesis and folding of derivatives of the μ-conotoxin SIIIA, the preclinical hormone hepcidin, and the trypsin inhibitor EETI-II. NMR and X-ray crystallography studies confirmed that these derivatives of disulfide-rich peptide retained the correct three-dimensional conformations. Moreover, the cystine-to-diaminodiacid replacement enabled structural tuning, thereby leading to an EETI-II derivative with higher bioactivity than the native peptide.*

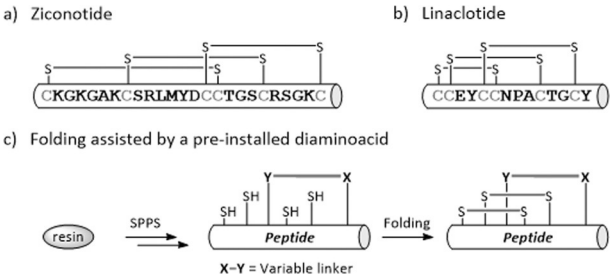

Figure 1. a) Ziconotide (approved in 2004 for treating severe and chronic pain). b) Linaclotide (approved in 2012 for treating irritable bowel syndrome with constipation and chronic idiopathic constipation). c) New derivatives of disulfide-rich peptides through cystine-to-diaminodiacid replacement.

Disulfide-rich peptides containing three or more disulfide bonds (e.g., neurotoxins, cyclotides) have emerged as promising molecules for diagnostic and therapeutic applications[1] because they exhibit excellent metabolic stability, strong bioactivity, and high target selectivity.[2] Two disulfide-rich peptides have already been approved as drugs (Figure 1a,b),[3] while more of them are in clinical trials or preclinical studies for diseases including pain disorders, cancer, bacterial/viral infections, acute heart failure, and coagulation disorders.[4] Chemical synthesis is a mainstream method for preparing disulfide-rich peptides for both medicinal chemistry research and industrial manufacture.[5] A common bottleneck with this approach is efficient formation of the correct pattern of disulfide bridges during folding.[6,7] Many approaches have been developed to reduce the problem, such as the use of orthogonal protecting groups[8] or partially directing of disulfide bonds.[9] Additional methods are needed to further improve the yields and reduce the time/labor costs.[10] An interesting recent finding was that a disulfide-to-diselenide replacement could significantly increase the folding efficiency of disulfide-rich peptides.[11] The explanation for this observation was that a diselenide bond, when generated first through selective oxidation, would decrease the number of intermediates during the subsequent folding process.[12] To date, the diselenide strategy has been applied to the synthesis of conotoxins[13] and other disulfide-rich peptides.[14] It has been suggested that the diselenide strategy may still lead to selenylsulfide formation and consequently to incorrect disulfide bridges.[15]

Herein, we report that the introduction of a single diaminodiacid bridge to disulfide-rich peptides with three or four disulfides can significantly improve their folding (Figure 1c). Our study was inspired by previous studies showing that diaminodiacids as disulfide surrogates can affect the stability or activity of bioactive peptides containing one to three disulfide bonds.[16] However, the effect (terms of folding efficiency, bioactivity, and structural validation) of diaminodiacid bridges on disulfide-rich peptides with three or four disulfides has not been studied before. Through experiments with three different disulfide-rich peptides, we have demonstrated that the diaminodiacid method is indeed practical and can be used to produce bioactive peptides with correct three-

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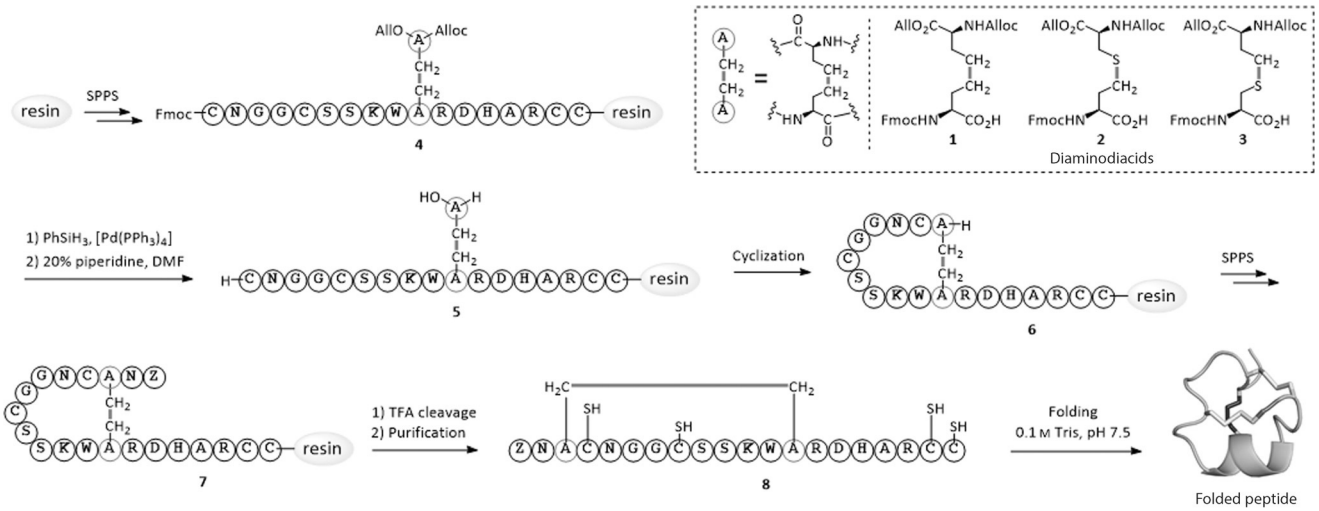


Figure 2. Synthetic route for preparation of SIIIA-1 with variable pre-prepared diaminodiacids. Z = pyroglutamate.

dimensional structures. Furthermore, since many different diaminodiacids can be used, the present method enables tuning of the bioactivity of disulfide-rich peptides in a new space.

Three representative diaminodiacids are shown in Figure 2. In these compounds, one α-amino group was protected with Fmoc (9-fluorenyl-methyloxycarbonyl), which enabled incorporation of the diaminodiacid into the peptide by using reported methods.[17] The other α-amino group and its neighboring acid group were protected with Alloc (allyloxycarbonyl) and allyl groups, which were stable during the subsequent Fmoc solid-phase peptide synthesis (SPPS). At the position where the cystine replacement needed to be installed, the Alloc and allyl groups were removed through treatment with $[Pd(PPh_3)_4]/PhSiH_3$. Intramolecular cyclization was then carried out between the acid group of the diaminodiacid and the terminal amino group of the growing peptide chain. The free α-amino group of the diaminodiacid was left intact in the cyclization step and could be used to complete the remaining peptide assembly.

To test the above synthetic route, we first examined μ-conotoxin SIIIA,[18] which has often been used as a model disulfide-rich peptide to study the folding process[11,13b] (Figure 3). We used the C–C-bridged diaminodiacid **1** to replace one of the three cystine residues and made three C–C bridged derivatives: SIIIA-1, SIIIA-2, and SIIIA-3. The yields of the isolated unfolded SIIIA-1, SIIIA-2, and SIIIA-3 peptides from Fmoc SPPS were 17%, 13%, and 11%, respectively. These values were comparable to the yield for native SIIIA (20%), thus indicating that the intramolecular cyclization step was highly efficient.

Next, the unfolded SIIIA, SIIIA-1, SIIIA-2, and SIIIA-3 were subjected to oxidative folding in a tris(hydroxymethyl)-aminomethane (Tris) buffer with air as the oxidizing agent. HPLC monitoring of the folding processes showed that SIIIA-1, SIIIA-2, and SIIIA-3 were folded more rapidly than SIIIA. Fewer peaks were observed for SIIIA-1, SIIIA-2, and SIIIA-3 compared to SIIIA, thus supporting our conjecture that the cystine-to-diaminodiacid replacement would reduce the number of folding intermediates. The outcome is an increase in the folding speed and yield, since accumulation of kinetically or conformationally trapped intermediates would limit the efficiency of oxidative folding.[19] Indeed, the HPLC yields for the folding of SIIIA-1, SIIIA-2, and SIIIA-3 were 81%, 79%, and 51%, respectively. These yields were higher than the folding yield for SIIIA, which was measure to be 49% by us and 50% in a previous study.[13b] Note that the diselenide-replaced analogue of SIIIA-2 was reported to have a folding yield of 80%.[13b] The C–C bridge thus exhibits the same effectiveness as the Se–Se surrogate in promoting the folding of SIIIA.

To verify that the diaminodiacid-replaced analogue maintained the correct three-dimensional conformation of SIIIA, we solved the solution structure of SIIIA-1 through a set of ^1H-^1H homonuclear solution NMR experiments (DQF-COSY, TOCSY, and NOESY). With a total of 202 NOE distance restraints (including 13 long-distance NOEs), 20 lowest-energy ensembles from 200 calculated structures were observed with an all heavy atom RMSD of 1.57 Å (Table S1 in the Supporting Information). The NOESY cross peaks and subsequently determined NMR structure (Figure 3 f) showed that SIIIA-1 maintained the correct Cys4–Cys19 and Cys8–Cys20 disulfide pairings (Figure 3). Similar chemical shifts for the Cα-H atoms were also observed for SIIIA[20] and SIIIA-1, except for the replaced Cys residues (Figure S10 in the Supporting Information), thus strongly indicating that replacement of the Cys3–Cys13 disulfide bond with a C–C-bridged diaminodiacid had little influence on the tertiary structure of SIIIA.

Having confirmed that the diaminodiacid-replacement method could improve the folding efficiency of μ-conotoxin, we next tested the method with a peptide with four disulfide bonds, namely, hepcidin.[21] This hormone regulates iron homeostasis by binding to ferroportin. Hepcidin dyssecretosis in vivo can cause many diseases (e.g., haemochromatosis) that can be treated with synthetic hepcidin or hepcidin analogues.[22] Direct oxidative folding of the hepcidin peptide is difficult because unfolded peptide and wrongly folded

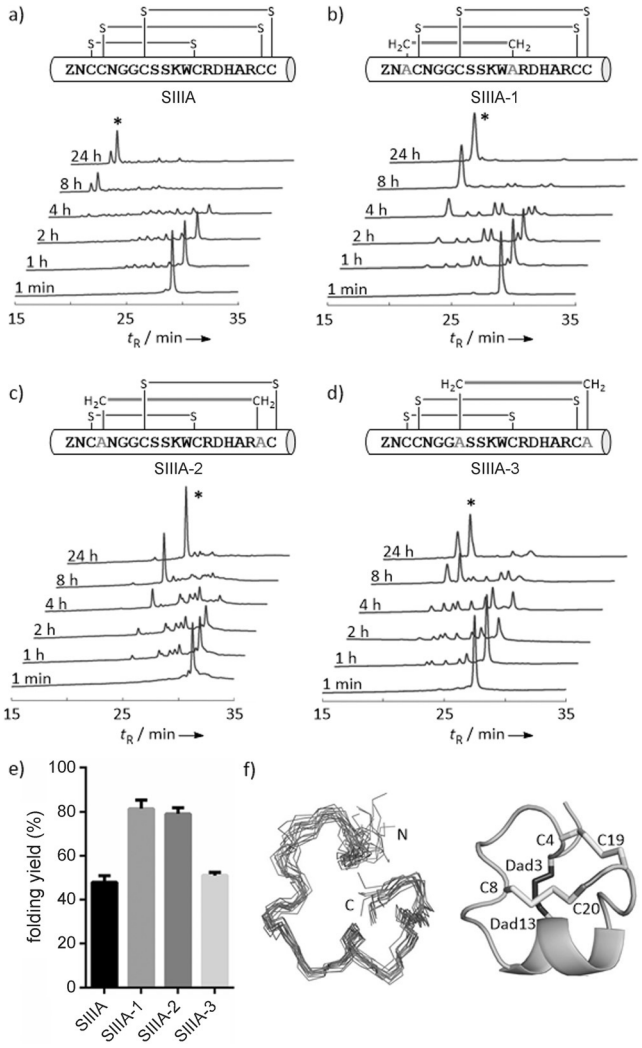


Figure 3. Structures and HPLC monitoring of the oxidative folding of SIIIA (a), SIIIA-1 (b), SIIIA-2 (c), and SIIIA-3 (d). Asterisks indicate the folded form. e) Folding yields (with error bars) determined as an average of three independent experiments. f) Backbone ensembles of 20 lowest-energy NMR structures of SIIIA-1 (left) and a ribbon representation of SIIIA-1 (right). The C–C bridge is shown in black while the two disulfide bridges are shown in pale gray. Dad = Diaminodiacid.

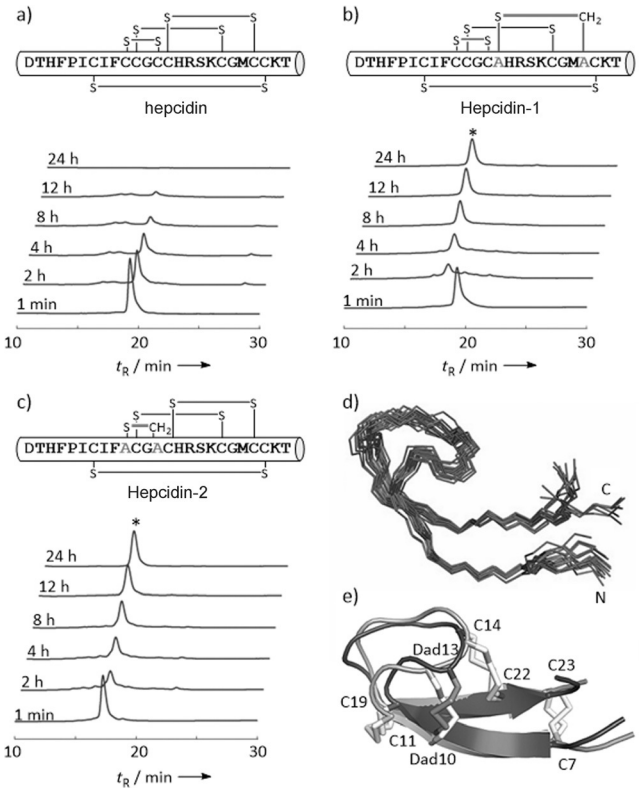


Figure 4. Structures and HPLC monitoring of the oxidative folding of hepcidin (a), Hepcidin-1 (b) and Hepcidin-2 (c). d) Backbone alignment of 20 lowest-energy structures of Hepcidin-2. e) Superposition of Hepcidin-2 (black) and the X-ray structure of hepcidin (grey; PDB ID: 3H0T). The S–C bridge is shown in dark gray and disulfide bonds in white.

intermediates tend to aggregate and precipitate in aqueous media. An acidic buffer and some special oxidants are needed to fold hepcidin with a folding yield of 12% (isolated product).[23] On the other hand, the folding yield for hepcidin under normal pH-neutral conditions was nearly zero (Figure 4a).

To test whether the diaminodiacid method can improve the folding of hepcidin, we synthesized two hepcidin derivatives in which either the Cys10–Cys13 or Cys14–Cys22 disulfide bond was replaced. We chose to use a different diaminodiacid with a S–C bridge to test its performance (Figure 4). Two target peptides (Hepcidin-1 and Hepcidin-2) was readily made through Fmoc SPPS with overall yields of 10% and 14% isolated product, respectively. Folding of these two peptides was conducted under the common neutral conditions (15% DMSO, 0.1M Tris, pH 7.5). HPLC monitoring showed that the folding of Hepcidin-1 and Hepcidin-2 proceeded rather smoothly to produce the folded products as a clean major peak in 24 h (Figure 4b,c). This observation was markedly different to that with native hepcidin under the same neutral conditions (Figure 4a). The HPLC yields for the folding of Hepcidin-1 and Hepcidin-2 were measured to be as high as 65% and 74% (yield of isolated product: 31% and 38%), which were significantly higher than the folding yield for hepcidin (12%[23]) under carefully optimized conditions.

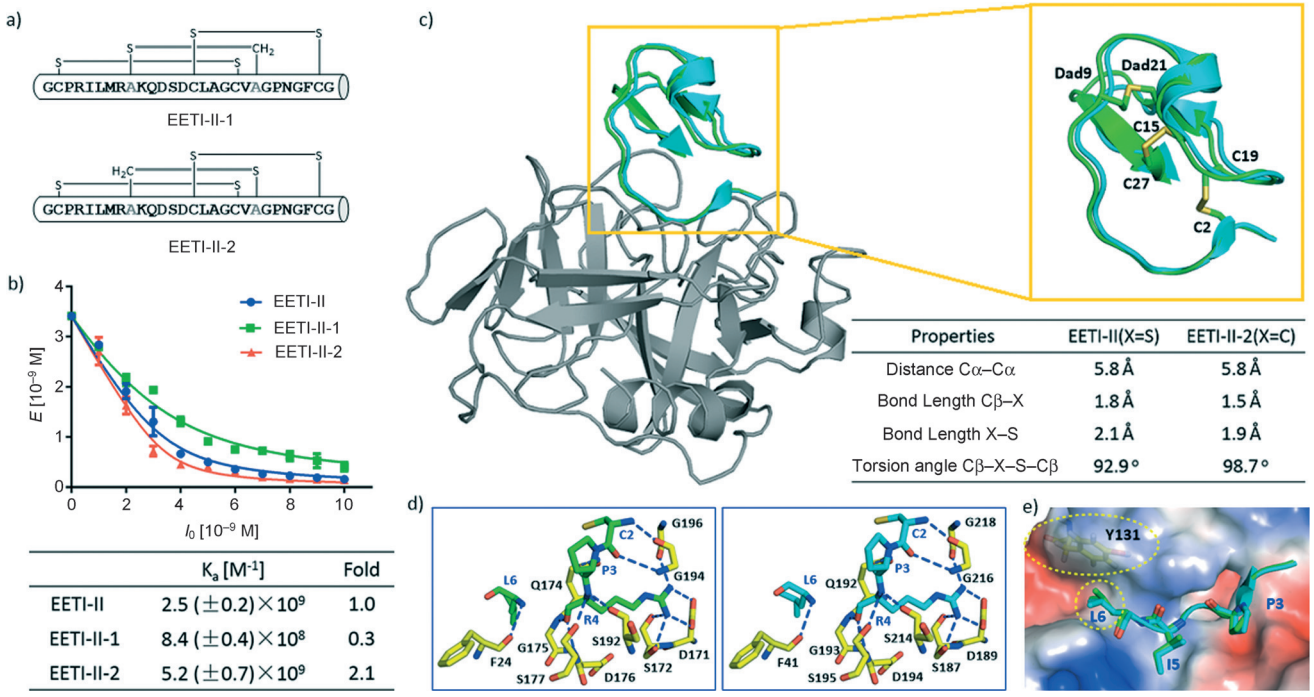
^{1}H-^{1}H homonuclear solution NMR experiments were carried out to resolve the three-dimensional structure of folded Hepcidin-2 through the use of standard DQF-COSY, TOCSY, and NOESY spectra. The 20 lowest-energy structures of Hepcidin-2 from 200 calculated structures showed a well converged conformation that maintained correct Cys7–Cys23, Cys11–Cys19, and Cys14–Cys22 disulfide bonds (Figure 4d). Hepcidin-2 was tightly folded with significant β-sheet character and a hairpin structure like native hepcidin[9a] (RMSD 0.65 Å for the β-sheet and 1.45 Å for the core regions). Furthermore, similar Cα-H chemical shifts (Figure S20) were observed for native hepicidin and Hepcidin-2, thus confirming that Hepcidin-2 maintained a similar three-dimensional structure to native hepcidin.[24]

The above two examples demonstrate that cystine-to-diaminodiacid replacement is truly effective in promoting the

folding of disulfide-rich peptides. Compared to the previous approaches (e.g., disulfide-to-diselenide replacement), the present method has one additional important advantage. That is, the bridges can be tuned with many potential options (e.g., C–C, C–N, C–O, and C–S bridges). Such tuning enables the generation of compounds with novel structures and sometimes, better bioactivity. To provide an example we studied the disulfide-rich peptide EETI-II,[25] a classic trypsin inhibitor with very strong activity. Two C–S-bridged EETI-II derivatives (EETI-II-1 and EETI-II-2) were made and they differed in the orientation of their C–S bonds (Figure 5a). Through Fmoc SPPS, the unfolded peptides of EETI-II-1 and EETI-II-2 were obtained with yields of 8% and 9% isolated product, respectively.

Oxidative folding of EETI-II, EETI-II-1, and EETI-II-2 was carried out by using the standard glutathione protocol (GSSG/GSH = 1:1, 1 mM) at pH 7.5. HPLC monitoring showed that the folding yields of EETI-II, EETI-II-1, and EETI-II-2 were 75%, 80%, and 85%, respectively, after 10 h (Figures S24, S27, S30). The trypsin inhibitory activity (K_a) values for EETI-II, EETI-II-1, and EETI-II-2 were then determined from the kinetics curves for the trypsin-mediated hydrolysis of Bz-Val-Gly-Arg-4-nitroanilide at different inhibitor concentrations (Figure 5b).[14a] For native EETI-II, the measured K_a value was $2.5 \times 10^9\,\text{M}^{-1}$. By comparison, the K_a values for EETI-II-1 and EETI-II-2 were measured to be 8.4×10^8 and $5.2 \times 10^9\,\text{M}^{-1}$, thus showing that the cystine-to-diaminodiacid replacement did not lead to a loss of activity. Since EETI-II-1 and EETI-II-2 only differ in the orientation of their C–S bridges, it is remarkable that such a change could alter the inhibitory activity by almost one order of magnitude. Furthermore, EETI-II-2 was more two-fold potent than native EETI-II, thus demonstrating that the cystine-to-diaminodiacid replacement could be beneficial not only for folding efficiency, but also to the bioactivity of disulfide-rich peptides.

To understand why EETI-II-2 was more active than EETI-II, we determined the crystal structure of the trypsin–EETI-II-2 complex. The complex was crystallized by using the sitting-drop vapor-diffusion method at 18°C. Good-quality crystals were obtained from a solution of trypsin and EETI-II-2 in 100 mM sodium citrate buffer at pH 5.6 containing 20% (v/v) 2-propanol and 20% (w/v) polyethylene glycol 4000.[26] The X-ray crystal structure (resolution = 1.3 Å) was then determined through the molecular replacement method by using the reported trypsin–EETI-II structure (PDB code: 1H9H) as a searching model. The overall structure showed that EETI-II-2 bound to trypsin in almost exactly the same manner as native EETI-II, with its N-terminal region inserted into the substrate binding pocket of trypsin (Figure 5c). Structure alignment of EETI-II-2 with the native EETI-II (mutation M7I) gave a fairly small RMSD value of 0.322 Å over 27 Cα atoms. Importantly, EETI-II-2 maintained the expected Cys2–Cys19 and Cys15–Cys27 disulfide bonds. The crystal structure provided evidence that the cystine-to-diaminodiacid replacement led to correctly folded peptides.



	K_a [M^{-1}]	Fold
EETI-II	2.5 (±0.2) × 10^9	1.0
EETI-II-1	8.4 (±0.4) × 10^8	0.3
EETI-II-2	5.2 (±0.7) × 10^9	2.1

Properties	EETI-II(X=S)	EETI-II-2(X=C)
Distance Cα–Cα	5.8 Å	5.8 Å
Bond Length Cβ–X	1.8 Å	1.5 Å
Bond Length X–S	2.1 Å	1.9 Å
Torsion angle Cβ–X–S–Cβ	92.9°	98.7°

Figure 5. Trypsin inhibitory activities of EETI-II, EETI-II-1, and EETI-II-2 and a crystal structure for trypsin in complex with EETI-II-2. a) Structures of EETI-II-1 and EETI-II-2. b) Trypsin inhibitory activities of EETI-II, EETI-II-1, and EETI-II-2. Bovine trypsin T1426 was used in the measurement. Error bars show the standard deviations calculated from three measurements. E = residual enzyme concentration. I_0 = concentration of the inhibitor. c) Crystal structure of trypsin (grey) in complex with EETI-II-2 (green) and comparison with native EETI-II (cyan; PDB code: 1H9H). d) Hydrogen-bonding network (blue) of EETI-II-2 (green) in complex with bovine trypsin (yellow), and native EETI-II (cyan) in complex with porcine trypsin (yellow). e) Hydrophobic interaction of trypsin with EETI-II-2 (green) and native EETI-II (cyan).

Some key structural parameters for native EETI-II and EETI-II-2 were measured (Figure 5c). It was found that the Cα–Cα distance (5.8 Å) in the Cys9–Cys21 bridge of native EETI-II was exactly the same as that in the corresponding diaminodiacid bridge of EETI-II-2. This finding is interesting because a previous study[27] has shown that the replacement of a disulfide bond with a diselenide bond could lead to a 0.3 Å variation of the Cα–Cα distance. The C–S-bridged diaminodiacid may thus better maintain the span distance of the disulfide bridge, possibly because of the combined effect of changes in the bond length and torsional angle. Indeed, the S–S bond length in the Cys9–Cys21 bridge of native EETI-II was measured to be 2.1 Å, while the C–S bond length in the corresponding diaminodiacid bridge of EETI-II-2 was 1.9 Å. At the same time, the C-S-S-C dihedral angle in the Cys9–Cys21 bridge of native EETI-II was measured to be 92.9°, while the C-C-S-C dihedral angle in the corresponding diaminodiacid bridge of EETI-II-2 was 98.7°.

Finally, although the Cys2 and Arg4 residues in EETI-II-2 presented very similar polar interactions with trypsin to those of native EETI-II (Figure 5d), Pro3, Ile5, and Leu6 in EETI-II-2 exhibited some observable difference in their conformations compared to EETI-II. This difference might be beneficial for the packing of hydrophobic amino acids in the groove in the trypsin surface because we found, for example, that the side chain of Leu6 in EETI-II-2 was closer to Tyr131 in trypsin than that of EETI-II (Figure 5e). Cystine-to-diaminodiacid replacement might thus cause a conformational change that enables better hydrophobic packing, which would explain the enhanced affinity of EETI-II-2 for trypsin.

To summarize, we have demonstrated that a single cystine-to-diaminodiacid replacement can significantly improve the folding efficiency of disulfide-rich peptides with three or four disulfides while maintaining their tertiary structures. Compared to the recently developed technology of diselenide replacement, the present method enabled the generation of bioactive peptides with many more structures that may be either more active or more selective. Our work uncovers interesting opportunities for the efficient production and optimization of disulfide-rich peptides for diagnostic and therapeutic applications.

Keywords: disulfide-rich peptide · peptide therapeutics · peptide synthesis · protein engineering · protein folding

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