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Backbone cyclization of a recombinant cystine-knot peptide by engineered Sortase A

Karen Stanger [a], Till Maurer [b], Harini Kaluarachchi [a], Mary Coons [b], Yvonne Franke [b], Rami N. Hannoush [a,*]

[a] Department of Early Discovery Biochemistry, Genentech, South San Francisco, CA, USA
[b] Department of Structural Biology, Genentech, South San Francisco, CA, USA

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ABSTRACT

Cyclotides belong to the family of cyclic cystine-knot peptides and have shown promise as scaffolds for protein engineering and pharmacological modulation of cellular protein activity. Cyclotides are characterized by a cystine-knotted topology and a head-to-tail cyclic polypeptide backbone. While they are primarily produced in plants, cyclotides have also been obtained by chemical synthesis. However, there is still a need for methods to generate cyclotides in high yields to near homogeneity. Here, we report a biomimetic approach which utilizes an engineered version of the enzyme Sortase A to catalyze amide backbone cyclization of the recombinant cyclotide MCoTI-II, thereby allowing the efficient production of active homogenous species in high yields. Our results provide proof of concept for using engineered Sortase A to produce cyclic MCoTI-II and should be generally applicable to generating other cyclic cystine-knot peptides.
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1. Introduction

Cyclotides are constrained cyclic peptides which contain a knotted arrangement of three disulfide bonds. The presence of the cyclized backbone and the cystine-knot framework in part make cyclotides remarkably stable to chemical degradation and proteolytic digestion [1–3], yet stability might be influenced by other factors such as shape and amino acid sequence. Initially discovered in plants, cyclotides were shown to exhibit a wide range of biological functions including oxytocic, insecticidal and antibacterial activities, to name a few [1,4]. Yet, how cyclotides are produced and circularized in nature has remained elusive. Recent data implicate asparaginyl endopeptidase as a potential enzyme which mediates transpeptidation between the N and C-termini of the cystine-knot peptide during the circularization process in plants [5–7]. While biochemical data using purified components to validate the proposed role of asparaginyl endopeptidase are still lacking, experiments in transgenic plants demonstrated that deletion of the gene encoding asparaginyl endopeptidase abrogated production of cyclotides [6,8], consistent with the notion that asparaginyl endopeptidase might be involved in backbone cyclization.

To date, there have been only a few reported methods for generating cyclic cystine-knot peptides in high yield and purity, with the most common method for head-to-tail cyclization being native chemical ligation [3,9,10]. More recent methods involve N → S acyl transfer reactions on polypeptide backbones containing N-terminal cysteine and a thiol-labile C-terminal Gly-Cys motif to generate circular miniproteins [10]. However, enzyme-mediated cyclization methods have started to garner attention due to their efficiency and cost effectiveness compared to synthetic methods. In one pioneering example, the enzyme trypsin was used to mediate peptide backbone cyclization of MCoTI [11], a cyclotide isolated from the seeds of the fruit *Momordica cochinchinensis* and shown to exhibit potent trypsin and matriptase inhibitory activity [2,12–16]. This method takes advantage of the inherent trypsin binding property of MCoTI-II to catalyze amide bond formation at a site that is recognized by trypsin and hence might not be suitable for generating variants of cyclotides that do not bind to trypsin.

Other methods to generate recombinant cyclotides have been described such as expressed protein ligation between a C-terminal thioester and an N-terminal cysteine residue [17,18] as well as intein-mediated protein trans-splicing in bacterial cells [19]. Despite these advances, there is still a need for versatile methods that involve less purification steps and enable efficient cyclization of cystine-knot peptides and their generation on a large scale in good yields. Here we report a biomimetic approach for producing

* Corresponding author.
E-mail address: hannoush.rami@gene.com (R.N. Hannoush).

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cyclotides by using an engineered version of the enzyme Sortase A to catalyze amide backbone cyclization. The method described herein utilizes an efficient cyclization reaction, which allows the production of active homogenous recombinant cyclotides in high yields and is generally applicable to generating other cyclic cystine-knot peptides.

2. Results and discussion

2.1. Biosynthesis of recombinant MCoTI-II

Our strategy for biosynthesis of cyclic MCoTI-II was to express a folded version of MCoTI-II followed by an enzyme-mediated transpeptidation reaction to form an amide bond between the N- and C-termini, thereby creating the cyclic peptide backbone. We selected the enzyme Sortase A, a transpeptidase from *Staphylococcus aureus*, which recognizes a consensus peptide sequence Leu-Pro-X-Thr-Gly (X = any amino acid residue). This enzyme cleaves the amide bond between Thr and Gly and catalyzes the ligation of the cleaved sequence to a Gly-Gly-Gly motif, resulting in a contiguous Leu-Pro-X-Thr-Gly-Gly-Gly polypeptide chain [20–22]. The Sortase A reaction which occurs primarily as an intermolecular reaction in *S. aureus,* has been recently exploited for generating circular proteins or creating fusions of different proteins, as well as labeling proteins at the N- or C-termini [23–25]. To generate cyclic MCoTI-II, we engineered a MCoTI-II construct (referred to as rMCoTI-II) that comprises a Gly-Gly-Gly motif at the N-terminus and Leu-Pro-Glu-Thr-Gly-Gly at the C-terminus (Fig. 1A). We also incorporated a 6×Histidine (His) and glutathione S-transferase (GST) tag N terminal to the Gly-Gly-Gly motif in order to utilize nickel nitrilotriacetic acid (Ni–NTA) affinity chromatography for purification and take advantage of the solubility of GST as a carrier protein (Fig. 1B). A Tobacco Etch Virus (TEV) cleavage site was also inserted between GST tag and the Gly-Gly-Gly motif in order to remove the His-GST tag after Ni–NTA purification (Fig. 1B) and enable subsequent cyclization by Sortase A. Using this strategy, rMCoTI-II was expressed in *Escherichia coli* and purified by Ni–NTA affinity chromatography (Fig. 1C). The eluted fractions were pooled and incubated overnight at 4 °C with TEV protease under oxidative conditions, which allowed folding of rMCoTI-II. A subsequent filtration step (MW cut-off 10 kDa) of the TEV reaction mixture trapped the His-GST tag while allowing rMCoTI-II to pass through. The filtrate was then collected and further purified by reversed-phase high performance liquid chromatography (RP-HPLC) to yield oxidized rMCoTI-II which was later used for optimization of the Sortase A-mediated cyclization reaction conditions (see below).

2.2. Sortase A-mediated cyclization of rMCoTI-II

While there could be great utility for the specific transpeptidase activity of Sortase A, the enzyme is significantly limited by poor reaction kinetics [26]. However, this limitation has recently been addressed by using a directed evolution strategy to select for Sortase A variants with improved catalytic activity [26]. This strategy led to the identification of four amino acid mutations P94S/D160N/D165A/K196T within the catalytic domain of Sortase A (Q60-K206) that, when combined, resulted in a 140-fold increase in k_{cat}/K_m. We generated this mutant Sortase A (referred to as m^4SrtA) and confirmed that the reaction kinetics were indeed significantly improved over the wild-type enzyme (Supp. Fig. 1). Therefore, the use of m^4SrtA would be advantageous for backbone cyclization of cystine-knot peptides due to potentially higher reaction yields compared to the wild-type enzyme. To our knowledge, engineered Sortase A has not been used for backbone cyclization of cyclotides.

We monitored the progress of the backbone cyclization reaction mediated by m^4SrtA by using liquid chromatography–mass spectrometry (LC–MS). Treatment of oxidized rMCoTI-II substrate (prepared above) with m^4SrtA under conditions which were optimized as described in detail below led to a loss of 132 atomic mass units as expected, owing to cleavage of two glycine residues on rMCoTI-II and loss of a water molecule from amide bond formation

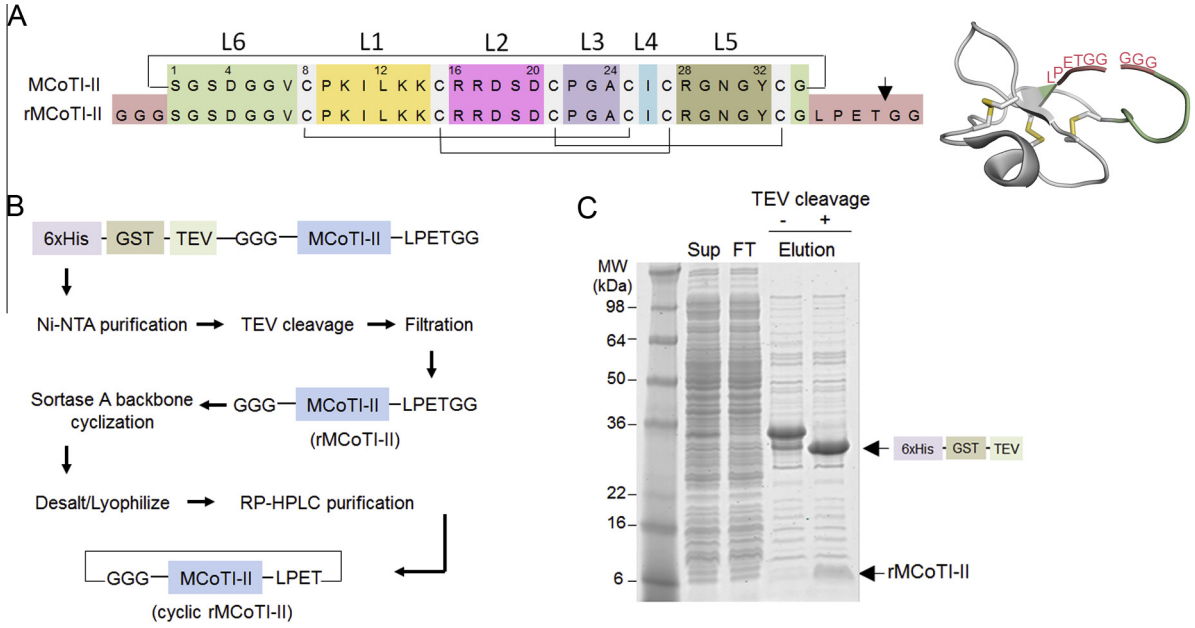

Fig. 1. Strategy for biosynthesis of MCoTI-II. (A) Primary structures of native and engineered MCoTI-II. Loop regions and the Sortase A recognition sequence are highlighted with disulfide bridges and the cyclic backbone (native) indicated. Cartoon was generated using MCoTI-II structure (PDB 1HA9) and the three disulfide bonds are highlighted; loop 6 is colored in green while the engineered Sortase A recognition sequence is shown in salmon. (B) Schematic of the purification and backbone cyclization method for generating cyclic recombinant MCoTI-II (rMCoTI-II). (C) SDS–PAGE showing purification progress of rMCoTI-II. The elution fractions from Ni–NTA column were pooled and subjected to TEV protease in redox buffer at 4 °C overnight. Sup, supernatant. FT, flow through.

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