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Butelase 1: A Versatile Ligase for Peptide and Protein Macrocyclization

Giang K. T. Nguyen, Antony Kam, Shining Loo, Anna E. Jansson, Lucy X. Pan, and James P. Tam*

School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, Singapore

S Supporting Information

ABSTRACT: Macrocyclization is a valuable tool for drug design and protein engineering. Although various methods have been developed to prepare macrocycles, a general and efficient strategy is needed. Here we report a highly efficient method using butelase 1 to macrocyclize peptides and proteins ranging in sizes from 26 to >200 residues. We achieved cyclizations that are 20,000 times faster than sortase A, the most widely used ligase for protein cyclization. The reactions completed within minutes with up to 95% yields.

Macrocyclization confers various desirable properties to peptides and proteins, including reduced conformational entropy, enhanced proteolytic stability, thermostability, and, for certain peptides, pharmacological potency and oral bioavailability.^{1–3} Large macrocycles are also well suited for inhibiting difficult targets, such as protein–protein interactions.⁴ Although most peptides and proteins exist in linear forms, macrocycles that range from 5 to 78 residues can be found in diverse organisms.⁵ These naturally occurring macrocycles exhibit high resistance to heat denaturation and proteolysis, and have inspired new efforts in protein engineering.^{6–8}

Various chemoenzymatic methods have been developed for macrocyclization. A commonly used chemical method is native chemical ligation for cyclizing cysteine-containing peptides through a proximity-driven S-to-N acyl migration.^{9,10} A major drawback of this method is the requirement of a cysteine residue at the N-terminus and a thioester linkage at the C-terminus, which limits their synthetic utility for noncysteine-containing peptides. Furthermore, chemical methods have become a challenge for macrocycles >100 residues. Enzymatic methods using a cyclase would provide an attractive solution.

Currently, only a few naturally occurring cyclases are known, including PATG, PCY1, and POPB.^{11–13} These cyclases exhibit restricted substrate specificity and can be used to cyclize small peptides of 5 to 11 residues. However, sortase A, which is a transpeptidase, and inteins, which are enzymes, have been applied successfully for the cyclization of large peptides and proteins, although they do not have innate cyclase functions and have several other limitations.^{8,14} Macrocyclization by sortase A requires a long reaction time (often an overnight incubation) and a high molar equivalent of enzyme (e.g., 0.1 to 10). Furthermore, sortase A has a long recognition sequence and leaves the pentapeptide LPXTG as an additional tag in the modified proteins. In contrast, inteins are autocatalytic splicing elements that require genetic fusion of a target protein with the intein

domain, which may affect protein folding or solubility.^{15,16} Thus, an efficient and site-specific ligase for head-to-tail macrocyclization of peptides and proteins would be highly desirable for protein engineering and drug discovery.

Recently, we discovered the fastest known ligase, termed butelase 1, from the tropical plant *Clitoria ternatea* that is widely grown as an ornamental, fodder, or medicinal plant.^{17–19} Butelase 1 is a naturally occurring cyclase involved in the biosynthesis of cyclotides, a family of plant cyclic cysteine-rich peptides.^{20–22} It presents in high yield (~1 mg/kg) and can be readily extracted from pods of *Clitoria ternatea*. Butelase 1 recognizes a tripeptide motif, Asn/Asp(Asx)-His-Val, at the C-terminus, and mediates peptide backbone cyclization by both cleaving the sorting sequence His-Val and ligating Asx to the N-terminal residue to form a macrocycle (Figure 1A). Previously, we demonstrated that butelase 1 efficiently cyclizes cyclotide precursors and small cysteine-rich peptides of about 2 to 3 kDa. Here we show that butelase 1 cyclizes noncysteine containing peptides and proteins >200 residues with excellent efficiency.

To demonstrate the general reactivity of butelase 1, we selected five noncysteine-containing peptide hormones ranging in size from 26 to 40 residues. They include human apelin, galanin, neuromedin U, and salusin α as well as rat neuromedin U. Human galanin and neuromedin U contain an intrinsic Asn residue, which allows “traceless” ligation that does not leave behind any additional sorting sequences in the final cyclized products. For the other three peptides, an additional Asn-His-Val sequence was added at the C-terminus and a Gly or Gly-Ile was introduced at the N-terminus as a linker sequence. Each cyclization reaction was performed in a reaction mixture containing 50 μ M peptide and 0.1 μ M butelase 1 (0.002 mol equiv) at 42 °C. High-performance liquid chromatography (HPLC) and mass spectrometry (MS) were used to monitor the reactions. Remarkably, butelase 1 achieved >95% cyclization yields within 5 min for all of the selected peptides (Figure 1B). We confirmed the Asn-ligation site and circular nature of each product using a tryptic or chymotryptic digestion and then analyzing the digested fragments by MS/MS (Figures S1–S5).

We next examined the cyclization kinetics of these peptide substrates. The apparent kinetic parameters for butelase 1 were calculated based on a Michaelis–Menten plot using GraphPad Prism (Figure 1E and Figure S6). We did not determine the kinetics for salusin α because of the coelution of its linear precursor and the cyclized product. The catalytic efficiencies were in the range of 1×10^5 to $1.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$. The greatest

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Figure 1. Butelase-mediated cyclization of noncysteine containing peptides. (A) Schematic illustration of a typical butelase-mediated cyclization. (B) MS profiles of peptide substrates before and after incubation with butelase 1. Reactions were performed at 42 °C for 5 min in the presence of 0.1 μM butelase 1 and 50 μM peptide substrates. (C) Enzymatic stabilities of cyclic and linear peptides against carboxypeptidase A. The degradation curve is similar for all five peptides, and only the graph for apelin is shown as a representative. (D) Angiogenesis activities of cyclic and linear apelin in the tube formation assay. (E) Kinetic parameters of butelase 1 for different peptide substrates.

catalytic efficiency reached $1.3 \times 10^6 M^{-1} s^{-1}$ for rat neuromedin U, which is twice as fast as what we observed in our previous study and $\sim 20,000$ times faster than sortase A ($\sim 50 M^{-1} s^{-1}$).²³ The slow kinetics of sortase A have prompted attempts to improve its catalytic efficiency by 140-fold using a directed evolution strategy.²⁴ However, even with the evolved variant of sortase A, cyclization of a 34-residue cyclotide McoTI-II remained slow and required the 0.5 mol equiv of sortase A and an incubation time of 72 h to reach 95% yield.²⁵ In contrast, the cyclization of peptides of a similar size in our present study required only 0.002 mol equiv of butelase 1 and a 5 min incubation time to reach 95% yield. Therefore, a typical butelase-mediated cyclization uses 250-fold less enzyme and completes 800-fold faster than a sortase A-mediated reaction, which can be translated to an approximate 200,000-fold improvement in the cyclization rate. However, a more accurate comparison of the catalytic efficiency of these enzymes would require the use of the same peptide substrate.

To show the effects of cyclization on proteolytic resistance against exopeptidases, we treated both linear and cyclized

peptides with carboxypeptidase A. These reactions were performed in the presence of 0.5 μM carboxypeptidase A and 25 μM of the tested peptides. We monitored the reactions by HPLC and MS. All linear peptides degraded within 5 min of incubation, whereas the cyclized forms were resistant to degradation and remained largely intact after 60 min (Figure 1C and Figure S7), suggesting that cyclization protects against exopeptidase-mediated degradation.

To demonstrate that bioactivity was maintained after macrocyclization, we used the endogenous angiogenesis factor apelin as an example in a tube formation assay. HUVEC-CS cells were seeded along with test samples and were incubated for 2 h. The wells were photographed, and the total number of junctions were quantified using ImageJ Angiogenesis Analyzer software. Both the cyclic and linear forms of apelin were equally active and induced ~ 2.5 -fold increase in tube formation (Figure 1D).

To establish the feasibility of butelase 1 for protein cyclization, we expressed three different recombinant proteins: green fluorescence protein (GFP), interleukin-1 receptor antagonist (IL-1Ra), and human growth hormone (somatropin); the latter

Figure 2. Butelase-mediated cyclization of protein substrates. (A) Schematic illustration of protein cyclizations using molecular models of GFP (generated from PDB code 1GFL), somatropin (PDB code 1HGU), and IL-1Ra (PDB code 1IRA). The cyclization reactions were performed for 15 min and monitored by SDS-PAGE. The cyclized GFP sample was run in duplicate. (B) Thermofluor assays for the linear and cyclic form of IL-1Ra. The melting temperature (T_m) shifted to a higher temperature upon cyclization. (C) Biological effects of native IL-1Ra (obtained from Peptotech), recombinantly expressed linear IL-1Ra with the Asn-His-Gly recognition sequence, and the cyclized IL-1Ra on IL-1 β induced ICAM-1 expression in A549 cells.

two proteins are used therapeutically. Each protein was constructed with an Asn-His-Val motif at the C-terminus and a linker Gly-Ile at the N-terminus. Each cyclization reaction was carried out in the presence of 25 μ M target protein and 0.1 μ M butelase 1 (0.004 mol equiv). The cyclization reactions were completed within 15 min and with >95% yields, as determined by SDS-PAGE and high-resolution ESI-MS (Figure 2A and Figures S8–S10). We also confirmed cyclization by tryptic or chymotryptic in-gel digestion and MS/MS analysis of the resulting fragments (Figures S11–S13). For comparison, sortase-mediated cyclization of GFP required a 24 h incubation and 1 mol equiv of sortase A.⁸ Thus, butelase 1 cyclizes GFP ~20,000 times faster than sortase A (Figure S14). It should be noted that cyclizations by sortase and split inteins have been

performed in cells and their *in vivo* cyclization rates *in vivo* are likely substantially different than their rates *in vitro*.^{26,27} These findings demonstrated that butelase 1 is an efficient ligase with promising potential uses, which may provide an orthogonal approach for the cyclization of peptides and proteins.

To determine whether butelase 1 can cyclize denatured proteins, somatropin and IL-1Ra were incubated at 95 °C for 1 min. Butelase 1 failed to cyclize both proteins after heat denaturation, which suggests that a properly folded protein conformation is required to maintain the N- and C-termini in close proximity for cyclization (Figure S15). The cyclization rate probably also depends on the accessibility of the N- and C-terminal residues. Unlike short peptides, which are generally conformationally flexible, the sterically hindered protein termini,

which may be buried inside the protein core, impede the cyclization reaction. Previously, we have demonstrated that the ligation yield for butelase-mediated ligation of ubiquitin was greatly improved after addition of a short linker peptide at the N-terminus.²⁸

To show that backbone cyclization improved thermostability, we performed a thermofluor assay on IL-1Ra and somatropin. The melting temperature (T_m) shifted to a higher temperature for IL-1Ra from ~53 to ~57 °C upon cyclization (Figure 2B). Our findings are consistent with those of previous studies in which cyclization enhances protein stability against heat denaturation.^{8,29,30} The T_m for somatropin was not determined because of a nonspecific unfolding curve (Figure S16). Furthermore, we also showed that the inhibitory activities of both linear and cyclized IL-1Ra on IL-1 β -induced ICAM-1 expression were comparable (Figure 2C and Figure S17). Native IL-1Ra from Peprotech was used as a positive control in our assay. IL-1Ra is an endogenous protein that binds to IL-1 receptors and antagonizes the effect of IL-1 β on ICAM-1 expression. Exposure to IL-1 β (10 ng/mL) increased ICAM-1 expression in A549 cells. Treatment with different forms of IL-1Ra abolished IL-1 β -induced ICAM-1 expression.

In conclusion, we developed butelase 1 for the macrocyclization of peptides and proteins. To our knowledge, butelase 1 is the first naturally occurring cyclase capable of cyclizing macrocycles >200 amino acids with high efficiency. Butelase 1 exhibits a broad substrate scope, a simple recognition motif, and fast kinetics, which allows it to generally complete cyclization reactions within minutes. Our method could open doors to industrial scale synthesis of artificial macrocycle libraries for novel pharmaceuticals and drug discovery. Furthermore, altering protein topology from a linear to a circular form represents a promising strategy for studies of protein structure, function, stability, and folding.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b11014.

Detailed experimental procedures and Figures S1–S17 (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*jptam@ntu.edu.sg

Notes

The authors declare no competing financial interest.

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