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Recombinant Expression of Backbone-Cyclized Polypeptides

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Abstract

Here we review the different biochemical approaches available for the expression of backbone-cyclized polypeptides, including peptides and proteins. These methods allow for the production of circular polypeptides either *in vitro* or *in vivo* using standard recombinant DNA expression techniques. Polypeptide circularization provides a valuable tool to study the effects of topology on protein stability and folding kinetics. Furthermore, having biosynthetic access to backbone-cyclized polypeptides makes the production of genetically-encoded libraries of cyclic polypeptides possible. The production of such libraries, which was previously restricted to the domain of synthetic chemistry, now offers biologists access to highly diverse and stable molecular libraries that can be screened using high-throughput methods for the rapid selection of novel cyclic polypeptide sequences with new biological activities.

Introduction

Backbone-cyclization or circularization (i.e. the covalent linkage of the of the N-terminal amino and C-terminal carboxylic groups) is a powerful tool for studying protein structure and function. Introduction of a covalent peptide bond between the N- and C-termini of a polypeptide provides a more stable constraint than, for example, disulphide bonds. In proteins where the C- and N- termini are in close proximity in the folded structure, which surprisingly is a common feature in single domain folded protein domains,¹ backbone-cyclization should decrease the entropy associated with the unfolded state of the protein, stabilizing its native folded state^{2,3} and therefore improving its biological activity.⁴ Protein circularization also provides a valuable tool to explore the effect that protein topology has on protein folding kinetics.^{2,5,6} Moreover, the introduction of covalent constraints into the polypeptide backbone, such as backbone-cyclization, should also make the corresponding polypeptide more resistant to proteolytic degradation, especially against exoproteases.^{7,8}

Backbone-cyclization is also widely used in bioorganic and medicinal chemistry to improve the biophysical and biochemical properties of biologically active linear peptides in the development of peptide-based therapeutics.^{7,8} Cyclization rigidifies the flexibility of the peptide backbone, creating constrained structural frameworks that often lead to higher biological activity and stability.^{2-4,7,8} Backbone-cyclized polypeptides, ranging from small peptides to small proteins, have also been found to be produced by bacteria, fungi, plants and more recently, by animals, where they feature a variety of biological roles.⁹ Many of these peptides, such as the immunosuppressant cyclosporine isolated from fungi and the antibiotic daptomycin isolated from bacteria, are produced by non-ribosomal peptide

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synthesis through large multienzyme complexes. Nonetheless, the number of backbone-cyclized polypeptides, which are produced in the ribosome as precursors and then post-translationally cyclized, is slowly increasing.⁹ For example, α -defensins (Fig. 1) are 18-residue circular peptides with broad spectrum antimicrobial activity, which are expressed in blood leukocytes and marrow of Old World monkeys,^{10,11} and so far represent the only circular peptides found in animals. Ribosomally synthesized backbone-cyclized peptides are also found in plants. Sunflower trypsin inhibitor 1 (SFTI-1, Fig. 1) is a bicyclic 14-residue peptide found in sunflower seeds with potent anti-trypsin activity.^{12,13} Cyclotides (Fig. 1), a novel family of small globular backbone-cyclized micro-proteins (~30 amino acids long), are also found in plants.¹⁴ Naturally-occurring cyclotides have been shown to possess several pharmacologically-relevant biological activities (see^{15,16} for recent reviews) and to be able to cross biological membranes^{17,18} thus making them attractive for the development of novel peptide-based therapeutics.^{19–21}

Recent advances in the fields of protein engineering and molecular biology have now made possible the biosynthesis of backbone-cyclized polypeptides using standard expression systems.^{22,23} The biological production (i.e. production of polypeptides mediated by ribosomal translation) of backbone-cyclized polypeptides offers many advantages over chemical synthesis. For example, although the chemical synthesis of polypeptides is generally limited to sequences no longer than 150–200 residues, biological approaches do not have such a limitation. Moreover, biological approaches also allow the generation of large libraries of cyclic peptides that are genetically encoded and can be rapidly screened inside living cells. This makes possible for the rapid selection of new peptide sequences able to attenuate or inhibit particular cellular processes in analogous way as the yeast two-hybrid technology works.²⁴

Several approaches that allow for the recombinant expression of backbone-cyclized polypeptides have been described so far (Figs. 1 & 2), and these include thioester-mediated ligation using expressed protein ligation (EPL)²⁵ or genetic re-programming,²⁶ intein-mediated protein trans-splicing²⁷ (please see²⁸ for an excellent searchable database of intein literature including circular peptides and protein) and protease-catalyzed traspeptidation.^{29,30} The present mini-review will describe the advantages and disadvantages of these approaches as well as their most recent applications (summarized in Table 1). It is worth to mention

Backbone cyclization using expressed protein ligation (EPL)

EPL is an extension of native chemical ligation (NCL), a chemoselective ligation approach used in the chemical synthesis of proteins from unprotected peptide segments.³¹ This reaction relies on the chemoselective reaction between the C-terminal thioester and an N-terminal Cys residue of two unprotected peptides. When these two groups are located in the same polypeptide chain, intramolecular NCL can be used for the generation of backbone-cyclized polypeptides.³²

Advances in protein engineering and the discovery of intein-mediated protein splicing have made possible the generation of recombinant proteins containing an N-terminal Cys residue and/or a C-terminal α -thioester functionality.²⁵ These important developments have, for the first time, made it possible to perform NCL reactions between recombinant and synthetic polypeptide fragments, therefore providing access to a multitude of chemically-engineered proteins, including backbone-cyclized polypeptides (see³³ for recent review of the different biotechnological applications of EPL).

The use of EPL for the recombinant production of backbone-cyclized polypeptides (Fig. 2A) was first reported by Camarero and Muir (1999) using the N-terminal SH3 domain of the c-

Crk as a model protein.⁴ In this work, the SH3 domain was fused to a modified VMA intein at the C-terminus and to the MIEGRC peptide motif (which contains a Factor Xa cleavage site) at the N-terminus. Once the precursor fusion protein was expressed in *E. coli*, purified and cleaved with Factor Xa, the resulting N-terminal Cys-containing SH3-intein fusion spontaneously reacted in an intramolecular fashion to produce a fully active circular SH3 domain.⁴ The cyclization process was very fast and efficient, which was attributed to the close proximity of the N-terminal Cys residue and the γ -thioester function in the folded state of the SH3 domain. Folding of the SH3 domain increases the local concentration of both reacting groups therefore facilitating the cyclization reaction. The same effect has been also observed in the cyclization of other small protein domains.^{34,35} Iwai and Plückthun have also used EPL for the backbone-cyclization of β -lactamase and GFP. In both cases the resulting circular proteins were biologically active and more stable.^{3,36} In-cell intramolecular EPL has been also used for the generation of a backbone-cyclized SH3 domain, providing the first evidence that this method can also be used in the complex cytoplasmic environment of a living cell.³⁷ This work opened up the possibility of production of genetically-encoded libraries that can be screened in the intracellular milieu of living cells for rapid selection of cyclic peptides with novel biological activities.

Our group has also recently reported the use of EPL for the recombinant *in vitro*³⁸ and in-cell³⁹ production of folded cyclotides using standard bacterial expression systems. This allowed us to generate a small genetically-encoded library on cyclotide MCoTI-I (Fig. 1) in which all the residues except Cys and those located in loop 6 were replaced by different amino acids.⁴⁰ The resulting library was screened for biological activity and the ability to adopt a native cyclotide fold. The results indicated that most of the residues in the MCoTI-cyclotide scaffold can be replaced without any significant detriment in the ability of the resulting sequences to fold correctly thus highlighting the robustness of this molecular framework for the generation of large genetically-encoded libraries of cyclotides.⁴⁰ These libraries could easily be screened in-cell for the rapid selection of novel cyclotide sequences with new biological activities.

Our group has also recently used in-cell EPL for the generation of other naturally-occurring backbone-cyclized polypeptides like SFTI-1⁴¹ and RTD-1⁴² (Fig. 1), and backbone-cyclized α -defensins,¹⁶ showing the general applicability of this method to other disulfide-rich circular polypeptide scaffolds.

Suga has recently reported the use of NCL for the generation of ribosomally translated backbone-cyclized polypeptides using cell-free expression systems.^{43,44} In this case the generation of the required γ -thioester functionality was accomplished by the incorporation of a cysteinyl-prolinyl glycolic acid ester moiety at the C-terminus of ribosomally produced polypeptides by reprogramming glycolic acid using a flexizyme system.^{44,45} The cysteinyl-prolinyl glycolic acid ester function can rapidly rearrange at physiological pH to produce a C-terminal thioester through the formation of a stable Cys-Pro diketopiperazine derivative.⁴⁶ This approach was used for the successful preparation of cyclic peptides SFTI-1 and RTD-1 (Fig. 1).⁴⁴ The same authors also showed that this approach can be used for the generation of libraries of cyclic peptides using the RTD-1 molecular framework. It should be noted, however, that due to the backbone-cyclized topology of these peptides, it is difficult to genetically-encode (for example using RNA-display approach) these libraries, making the screening of such libraries a challenging task. This limits the complexity of the libraries that can be generated using this approach. The low yields usually associated with cell-free expression systems also limit the utility of this method for the screening of large libraries of backbone-cyclized polypeptides.

Backbone cyclization using intein-mediated protein *trans*-splicing (PTS)

Intein-mediated PTS is another method that can be used for the ribosomal preparation of backbone-cyclized polypeptides (Fig. 2B). PTS is a post-translational modification similar to protein splicing with the difference that the intein self-processing domain is split into N- (I_N) and C-intein (I_C) fragments. The split-intein fragments are not active individually, however, they can bind to each other with high specificity under appropriate conditions to form an active protein splicing or intein domain in *trans*.²² PTS-mediated backbone-cyclization can be accomplished by rearranging the order of the intein fragments. By fusing the I_N and I_C fragments to the C- and N-termini of the polypeptide for cyclization, the *trans*-splicing reaction yields a backbone-cyclized polypeptide (Fig. 2B). This approach was first reported by the Benkovic group using the naturally occurring *Synechocystis* sp. (*Ssp*) PCC6803 DnaE split intein, and it has been used for the preparation of many small backbone-cyclized polypeptides as well as large genetically-encoded libraries of small cyclic peptides in bacterial cells.^{47–49} Payan has also used this split intein to generate libraries of random cyclic peptides in the cytoplasm of human cells using a retroviral expression vector.⁵⁰ These libraries were screened in-cell for modulation of the IL-4 signaling pathway leading to the selection of several cyclic peptides able to selectively inhibit the γ promoter. More recently, Schultz and Benkovic have used this approach in combination with nonsense codon suppressor tRNA technology to build large libraries of cyclic hexapeptides using an expanded set of non-natural amino acid building blocks.⁵¹ These libraries were screened in-cell for selecting HIV-protease inhibitors using a lethality reporter. This work produced several HIV protease inhibitors with IC_{50} values in the low micromolar range.⁵¹

PTS has also been used for the production of recombinant backbone-cyclized proteins. In a recent study, the artificially split *Spp* PCC6803 DnaB mini-intein was used for the cyclization of the TEM-1 β -lactamase in the bacterial periplasm.⁵² This was achieved by adding the TEM-1 β -lactamase native periplasm export signal to the intein precursor protein. The same authors also used this approach to generate a genetically-encoded library of small cyclic peptides and estimated that ~50% of the members of the library were able to cyclize efficiently.⁵²

The use of the *Ssp* DnaE or DnaB split inteins requires the presence of specific amino acids at both extein-intein junctions for efficient protein splicing to occur.²⁷ To overcome this limitation, our group has recently used the *Nostoc punctiforme* PCC73102 (*Npu*) DnaE split-intein⁵³ for the production of backbone-cyclized polypeptides.⁵⁴ This DnaE intein has the highest reported rate of protein *trans*-splicing ($t_{1/2} \sim 60$ s) and also has a high splicing efficiency.^{55,56} We used this approach for the in-cell production of cyclotide MCoTI-I in bacterial cells.⁵⁴ We found that in-cell expression of folded MCoTI-cyclotides (Fig. 1) by PTS was about 7 times more efficient than intramolecular EPL-mediated backbone-cyclization, thereby providing an attractive alternative for the production of these types of polypeptides in bacterial cells. This improvement could be explained by the use of the split-intein *Npu* DnaE, which is extremely efficient and exhibits fast kinetics.^{53,56} It is also worth noting that in contrast with the *Ssp* DnaE and DnaB inteins, which require three to four native residues at the N- and C-terminal extein-intein junctions to work efficiently,⁵⁷ the *Npu* ortholog used in this work was able to tolerate different sequences at both junctions, as demonstrated by the efficient *trans*-splicing of the MCoTI-*Npu* DnaE intein precursor.⁵⁴ The high efficiency of *Npu* DnaE PTS-mediated cyclization combined with the use of nonsense suppressing orthogonal tRNA/synthetase technology made possible the in-cell production of cyclotides containing unnatural amino acids (Uaas) possible.⁵⁴ This approach was used to incorporate *p*-azido-phenylalanine, which can react efficiently through copper-free click chemistry with dibenzocyclooctyne-containing fluorescent probes to provide in-cell

fluorescently-labeled cyclotides.⁵⁴ This finding opens the possibility for *in vitro* and potentially also in-cell screening of genetically-encoded libraries of cyclotides for the rapid selection of novel cyclotide sequences able to bind a specific bait protein using high throughput cell-based optical screening approaches.

Backbone-cyclization using protease-catalyzed transpeptidation

Protease-catalyzed transpeptidation is commonly used by prokaryotes to attach polypeptides to peptidoglycan in their cell wall envelope.⁵⁸ For example, sortases are transpeptidase enzymes found in most Gram-positive bacteria that are specialized in this task. The *Staphylococcus aureus* sortase A (SrtA) is one of the most widely used for biotechnological applications. SrtA specifically recognizes peptide sequences containing the LPXTG (where X can be any residue) motif, cleaving the peptide bond between the Thr and Gly residues. The SrtA active site contains a Cys residue that generates a transient thio-acyl enzyme intermediate during the cleavage reaction. This branched thioester intermediate then reacts with the α -amino group of poly-Gly (a penta-Gly sequence in the case of SrtA) substrates. In the absence of any poly-Gly substrate the thioester group is simply hydrolyzed.

SrtA has been recently used for the generation of circular GFP using an intramolecular version of sortase-mediated ligation (SML) (Fig. 2C).^{29,30} Other proteins/polypeptides cyclized using SML include dihydrofolate reductase (DHFR) and pleckstrin homology (PH) domains,⁵⁹ as well as circular versions of cytokines and histatin 1.⁶⁰

SML backbone-cyclization has not been shown to work in-cell yet, which limits its use for the generation of genetically-encoded libraries of cyclic peptide for genetic experiments. The use of sortases also requires the use rather large recognition sequences (LPXTG₅ in the case of SrtA), which remain incorporated in the final circular polypeptide and therefore it may affect that applicability of this approach in some applications, for example the synthesis of small cyclic peptides.

Serine proteases have been recently used for “traceless” backbone-cyclization of polypeptides. Craik has reported the backbone-cyclization of the naturally occurring cyclic peptide SFTI-1 (Fig. 1) using trypsin.⁶¹ A similar approach has been also reported for the enzymatic cyclization of MCoTI-cyclotides.⁶² MCoTI-cyclotides and SFTI-1 are both potent trypsin inhibitors with K_i values ranging in the picomolar to nanomolar range, respectively. In both cases the enzyme trypsin was able to catalyze the formation of a peptide bond between the α -amino and α -carboxylic groups of the P1' and P1 residues, respectively. Recent evidence also suggests that the biosynthesis of cyclotides and SFTI-1 involves an asparaginyl endopeptidase-mediated transpeptidation step during the cyclization process of these circular peptides in plants.^{63,64} Complete elucidation of the mechanism how these peptides are produced in plants should open the possibility in the near future for the production of particular circular polypeptides using genetically engineered plants.

Conclusion and Remarks

We have briefly reviewed different biochemical methodologies for the production of backbone-cyclized polypeptides ranging from small polypeptides to proteins. These include the use of thioester-mediated ligation employing either modified inteins^{38,40} or genetic code reprogramming,^{43,44} and protein splicing mediated by inteins^{27,54} or proteases.^{61,62}

The production of backbone-cyclized polypeptides using standard DNA recombinant approaches has many biotechnological applications. For example, we have described several methods that allow in-cell production of cyclic polypeptides. This makes possible the generation of large genetically-encoded libraries of cyclic peptides inside live cells that can

be rapidly screened for the selection of novel sequences able to modulate or inhibit the biological activities of particular biomolecular targets.^{27,50,51} The use of PTS in combination with nonsense suppressing orthogonal tRNA/synthetase technology has also made possible the introduction of non natural amino acids onto in-cell expressed backbone-cyclized polypeptides.^{51,54} Expansion of the genetic code allows for the incorporation of novel chemical functionalities that can be used in molecular evolution and cell based assays for the selection of novel cyclic peptides with improved biological or novel biophysical activities.^{51,54}

Having access to the recombinant production of cyclic polypeptides also facilitates the introduction of NMR active isotopes such as ¹⁵N and/or ¹³C in a very inexpensive fashion.^{16,42,65,66} This should facilitate the use of heteronuclear NMR spectroscopy to study structure-activity relationships of any biologically active circular polypeptides and their molecular target.^{65,66}

In summary, the development of these new biochemical approaches for the production of cyclic peptides in biological systems presents new and exciting opportunities that before were restricted only to synthetic chemists. The production of genetically-encoded libraries of constrained cyclic peptides in cells or even in whole organisms should offer biologists with a unique opportunity to perform molecular evolution experiments to be able decode complex biochemical pathways and protein interaction networks present in living cells.

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Figure 1.

Summary of the most common biological approaches used for the production of backbone-cyclized polypeptides using standard DNA techniques. All of these approaches rely on the ribosomal synthesis of a precursor protein that is then processed to provide the corresponding backbone-cyclized polypeptide. Three examples of naturally-occurring backbone-cyclized polypeptides are also shown. MCoTI-II is a cyclotide with trypsin inhibitory activity isolated from the seeds of a tropical squash (*Momordica coconchinesis*) (Protein data bank code: 1IB9). RTD-1 is a primate θ -defensin with broad antimicrobial activity (Protein data bank code: 1HVZ). SFTI-1 is a potent trypsin inhibitor isolated from sunflower (*Helianthus annuus*) seeds (Protein data bank code: 1JBN). All three peptides have been produced in biological systems (See Table 1).

Figure 2.

Scheme representing the mechanisms for the most common approaches for the biological production of backbone-cyclized polypeptides using EPL (A), PTS (B) and SML (C). The terms I_C and I_N represent the C- and N-intein fragments of a split intein, respectively. Figure adapted from reference.²³

Table 1

Examples of backbone-cyclized polypeptides, including peptides and proteins, produced by using EPL, PTS, SML, protease-mediated-ligation and genetic reprogramming.

Protein/Peptide	Polypeptide length	Method used	Intended use of cyclic product/s	Reference
HIV protease inhibitor	6	PTS	Library scaffold	51
Pseudostellarin F	8	PTS	Natural product	27
SFT-1	14	EPL	Natural product and library scaffold	41
		Genetic reprogramming	Natural product	44
		Trypsin-mediated cyclization	Natural product and NMR structure	61
RTD-1	18	EPL	Antimicrobial activity	42
		Genetic reprogramming	Natural product and library scaffold	44
Crp4	32	EPL	Antimicrobial activity and stability	16
MCoTI-I/II	34	EPL	Library scaffold	38,39,40
		PTS	Incorporation of unnatural amino acids	54
		Trypsin-mediated cyclization	Production of protease inhibitor/s	62
Histatin 1	38	SML	Biological activity	60
c-Crk SH3	57	EPL	Biological activity	4
		EPL	Folding kinetics	2
		EPL	In-cell synthesis	37
Acyl carrier protein	76	PTS	Stability	67
DnaB helicase	121	PTS	Stability	68
Firefly luciferase	160	PTS	Screening system	69
LIM-only protein 4	195	PTS	Stability	70
GFP	238	SML	Stability	29,30
		PTS	Stability	3
TEM 1 -lactamase	263	PTS	Stability	52
		EPL	Stability	35
Glucose transporter	277	PTS	Stability	71