Protein Engineering Hot Paper

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Generation of a Genetically Encoded, Photoactivatable Intein for the Controlled Production of Cyclic Peptides**

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Abstract: *Cyclic peptides are important natural products and hold great promise for the identification of new bioactive molecules. The split-intein-mediated SICLOPPS technology provides a generic access to fully genetically encoded head-to-tail cyclized peptides and large libraries thereof (SICLOPPS = split-intein circular ligation of peptides and proteins). However, owing to the spontaneous protein splicing reaction, product formation occurs inside cells, making peptide isolation inconvenient and precluding traditional in vitro assays for inhibitor discovery. The design of a genetically encoded, light-dependent intein using the photocaged tyrosine derivative ortho-nitrobenzyltyrosine incorporated at an internal, non-catalytic position is now reported. Stable intein precursors were purified from the E. coli expression host and subsequently subjected to light activation in vitro for both the regular protein splicing format and cyclic peptide production, including the natural product segetalin H as an example. The activity of the intein could also be triggered in living cells.*

Cyclic or conformationally constrained peptides represent an important class of bioactive molecules. Many compounds derived either from natural sources or from man-made design are used in therapy or being tested in clinical trials.[1] Natural products include the nonribosomal peptides[2] as well as ribosomally synthesized compounds that undergo post-translational modification. The latter can contain disulfide bridges and other cross-linking bonds to achieve rigidification[3] or be truly head-to-tail cyclized.[4] Cyclic peptides are privileged structures for inhibitor design because their rigidity off-sets an energetic loss of the entropic contribution when binding to a protein target, in comparison to the more flexible linear counterpart. Additional advantages are increased proteolytic stability and membrane permeability. Their large size and protein-like structure make cyclic peptides particularly promising candidates for the inhibition of protein–protein interactions.

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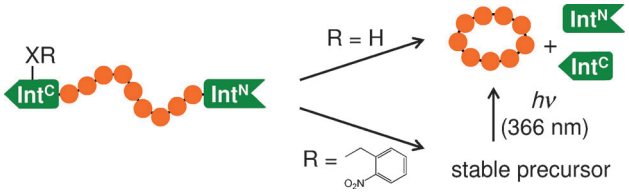
The identification of new bioactive cyclic peptides as peptidic lead compounds or as research tools is a pressing goal.[1,5] Genetically encoded peptides are of particular interest for lead compound identification because they easily lend themselves to the production of large libraries by randomization on the DNA level. Various technologies have been developed to cyclize or conformationally restrict ribosomally synthesized peptides, for example by intein-[6] or sortase-mediated[7] ligation, split-intein circular ligation of peptides and proteins (SICLOPPS; Scheme 1),[8] by disulfide



Scheme 1. The split-intein-mediated cyclization of a polypeptide by the SICLOPPS technology is a spontaneous reaction (top).[8a] The approach described herein blocks protein splicing by the incorporation of a photolabile protecting group and thereby allows isolation of the unspliced precursor (bottom). X = amino acid side chain.

formation[9] or cysteine cross-linking,[10] and by the incorporation of functional handles.[6b,11] Strikingly, only very few of these methods provide access to truly head-to-tail cyclized products, also because having at least one free terminus of the peptide backbone in an otherwise branched cyclic structure allows for anchoring the compound to a platform useful for selection, such as a phage[9] or mRNA display.[5c] In contrast, a head-to-tail cyclized peptide has no termini and cannot be anchored to its encoding genotype in a straightforward way. For example, the SICLOPPS technology to produce large cyclic peptide libraries (Scheme 1) allows powerful selections in living cells,[12] but is also severely limited by the fact that the non-covalent genotype–phenotype coupling is lost once the cellular envelope is disrupted. However, for many protein targets, a selection system cannot be established inside the cell and also the control of parameters, such as concentration, exposure time, and buffer conditions, inside the cell is severely limited. Furthermore, the purification of cyclic peptides from a cell extract is very challenging.

Herein, we aimed to address this problem by developing a novel, fully genetically encoded intein that can be activated by light. We hypothesized that with a light-dependent intein, the protein splicing reaction would be blocked, and the unspliced precursor could be purified from the cell extract

before the intein is triggered by light and formation of the cyclic peptide occurs according to the SICLOPPS method.

Previously, various approaches to render the otherwise spontaneous protein splicing reaction controllable by an external trigger have been undertaken. These efforts included fusion of the intein with a small-molecule binding domain[13] or a light-responsive protein unit,[14] and the use of temperature-dependent[15] or redox-sensitive inteins.[16] Other approaches explored split intein systems in which one of the fragments was chemically synthesized and contained a photochemically labile protection group positioned in a way that only the deprotected fragment gave rise to intein activity.[17] However, none of the mentioned systems has been used for the in vitro generation of cyclic peptides, partly because a two-fragment split intein strategy is not compatible with the SICLOPPS method for making cyclic peptide libraries.

To design a suitable intein with a light-dependent switch, we turned to the amber stop codon suppression technology, which allows for the genetic encoding of several photoprotected amino acids.[18] Although the incorporation of photoprotected Cys or Ser monomers into a catalytic position at the splice junction of an intein seemed to be straightforward for a semi-synthetic intein[17c] and was previously reported using a cell-free translation system,[19] we dismissed this route because at the beginning of this study, no system was available to encode photoprotected Cys or Ser monomers in *E. coli*.[20] Rather, our design strategy was to create a steric perturbation at a non-functional, but structurally critical position in the intein that can be relieved by removal of the photoprotection group with light (Scheme 1). With this idea, we turned to *ortho*-nitrobenzyl-protected tyrosine (ONBY), which can be incorporated both in *E. coli* and in mammalian cells (Figure 1 A).[21] We chose the M86 mutant of the *Ssp* DnaB intein (named M86 intein hereafter) because it is a well characterized intein that has undergone significant optimization through a directed evolution procedure.[22] It displays a high tolerance towards the flanking amino acids, which is an important prerequisite for the generation of arbitrary peptides and peptide libraries. The M86 intein contains two Tyr residues, which are both located on the surface of the protein (Supporting Information, Figure S1 A).[23] Thus, a structural alteration at these positions was not expected to have a significant effect on folding. We therefore evaluated the positions of three phenylalanine residues as well as that of one isoleucine residue, all of which were part of the hydrophobic core (Figure S1 B). Intein variants with the amino acid substitutions F28Y, F75Y, F145Y, I2F, and I2Y were created by site-directed mutagenesis in the model construct MBP–M86intein–Trx. Figure S2 shows that the mutation F28Y had no obvious effect on protein splicing. For F145Y, increased N-terminal cleavage was observed. The F75Y mutation led to no obvious formation of side products, but showed partial accumulation of the unspliced precursor, indicating that the rate of the splicing process was reduced. The I2F mutant still supported splicing, but the I2Y mutant was nearly completely inactive and thus not useful for our approach. On the basis of these results, positions 28, 75, and 145 were further advanced for incorporation of ONBY.

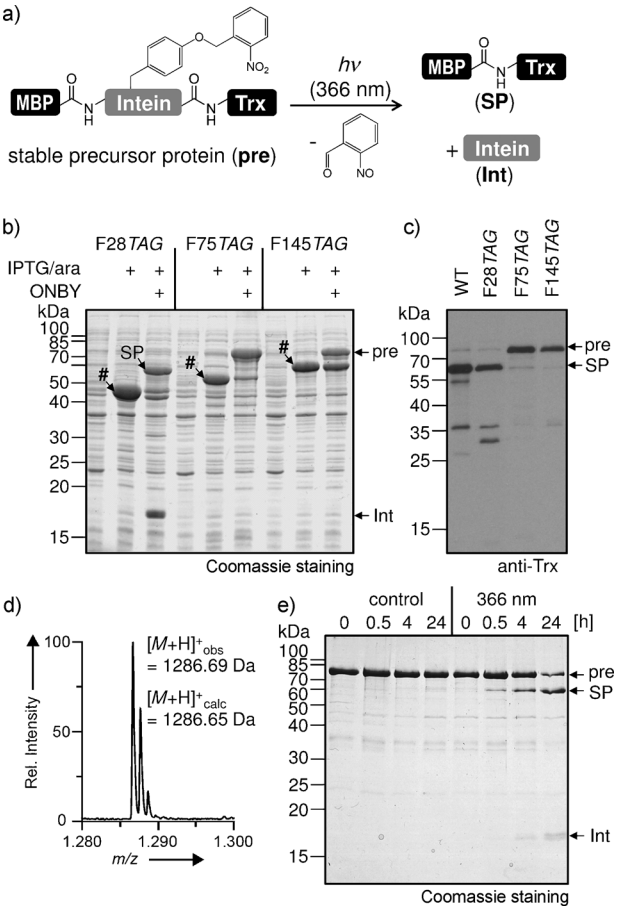


Figure 1. Generation of a photoactivatable intein. A) Model reaction to monitor protein splicing with ONBY at various positions of the intein. B) Analysis of whole-cell extracts from *E. coli* cells incorporating ONBY at the indicated positions. # = truncation product resulting from termination at the amber stop codon. C) Western blot analysis of samples as in (B) of the intein-ONBY variants in comparison with the wild-type (WT) construct. D) MS analysis of the tryptic fragment XLTIDGWKR (X = ONBY) prepared from the F75ONBY-intein precursor. E) Purified F75ONBY-intein precursor irradiated at $\lambda = 366$ nm for 4 min. Splice product formation was monitored at the indicated time points after irradiation. ara = arabinose, SP = splice product.

Figure 1 B shows that ONBY incorporation was successful at all three positions of the intein using the same model protein. The F28ONBY substitution produced only spliced proteins in the whole-cell extract indicating that the structural modification at this position was not sufficient to block intein activity. In contrast, the variants with F75ONBY and F145ONBY were very promising because only the full-length unspliced precursors could be observed (Figure 1 B, C). These two candidates where then further tested in a similar model protein with an additional C-terminal His_6 tag to allow for efficient precursor purification (MBP–intein(ONBY)–Trx–His_6). Figure S3 shows that the precursor with F145ONBY was not fully stable during the purification procedure.

Much to our delight, the precursor with F75ONBY proved to be completely stable under the purification conditions and could be easily prepared in larger quantities.

Mass spectrometric analysis confirmed incorporation of the unnatural amino acid (Figure 1D). With UV irradiation (366 nm) for four minutes, protein splicing could be induced and proceeded to approximately 70% conversion. Again, no formation of N- or C-terminal cleavage products could be observed. A closer biochemical investigation of the precursor MBP–intein(F75ONBY)–Trx–His_6 showed it to be susceptible to N-terminal cleavage at higher concentrations of nucleophiles such as DTT (DTT = dithioerythritol), despite its stability at pH 7 in the absence of nucleophiles (Figure S4). Thus, the blocked intein precursor was still capable of rearranging from the peptide bond into the thioester at the upstream scissile bond, suggesting that its structure is only locally perturbed by the ONBY incorporation. We termed this stable and photo-inducible intein precursor ONBY75-intein. Importantly, protein splicing with the ONBY75-intein could also be triggered in living *E. coli* cells (Figure S5), indicating the potential for in vivo applications.

We then turned to the exploitation of the ONBY75-intein for the controlled production of cyclic peptides. An expression vector for an intramolecular arrangement of the intein split after position 11[22] was designed. The encoded protein SBP–Int^C(ONBY75)–peptide–Int^N–His_6 also contained affinity tags for efficient purification (SBP = streptavidin-binding peptide). We first designed an artificial sequence for a 13-mer peptide (SNGQTGGAPRWGH; see Figure 2). The precursor could be expressed in *E. coli* and purified in stable form. Mass spectrometric analysis confirmed the incorporation of the unnatural ONBY amino acid (Figure S6). As for the cis-splicing construct, intein activity could be efficiently triggered by irradiation for four minutes (366 nm) and proceeded to 80–90% conversion (Figure 2C). Analysis of the reaction mixture by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) revealed the presence of the cyclic 13-mer peptide. Only a very weak signal for the linear peptide was detectable indicating that the desired cyclization through intramolecular protein trans-splicing was very efficient compared to potential cleavage side reactions (Figure 2D). To further investigate the generality and versatility of our approach, we also studied the production of a bioactive natural product. Segetalins are short cyclic peptides that are produced by higher plants.[4a] Segetalin H was isolated from the seeds of *Vaccaria segetalis* and is a head-to-tail cyclized pentamer, cyclo(SGYRF), with estrogenic activity.[25] Figure 3 shows that the respective split

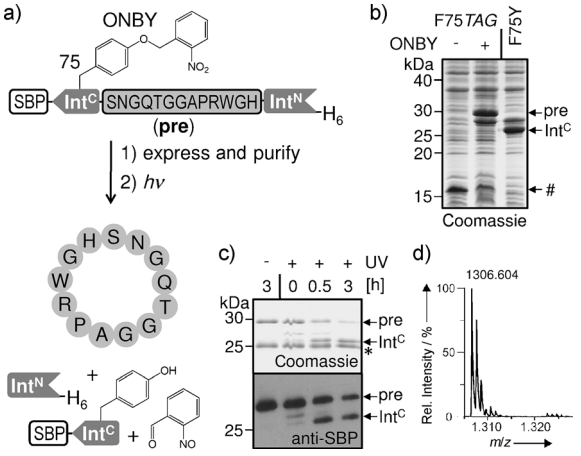


Figure 2. Photocontrolled production of a cyclic 13-mer peptide. A) Experimental procedure. B) ONBY precursor (pre) expression analyzed in a whole-cell extract from *E. coli* cells, with F75Y control for comparison. C) Photoinduced conversion of the stable, purified precursor (anti-SBP blot performed with streptavidin-coupled horseradish peroxidase). *: The co-purified protein was identified as *E. coli* protein SlyD, a typical Ni-NTA contamination.[24] D) MALDI-TOF MS analysis of the reaction mixture after irradiation confirms the presence of the cyclic peptide ($[M+H]^+_{obs}$ = 1306.604 Da, $[M+H]^+_{calc}$ = 1306.604 Da; linear peptide: $[M+H]^+_{calc}$ = 1324.614 Da).

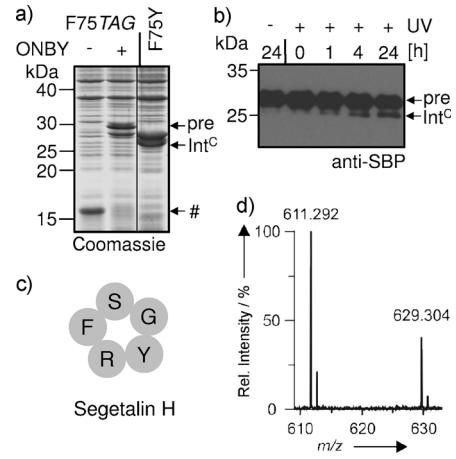


Figure 3. Photocontrolled production of segetalin H. The sequence SGYRF was expressed in the same format as shown in Figure 2A for the 13-mer. A) ONBY precursor (pre) formation analyzed in a whole-cell extract from *E. coli* cells, with F75Y control for comparison. B) Photoinduced conversion of the stable, purified precursor. Shown is an immunoblot using streptavidin-coupled horseradish peroxidase. C) Structure of segetalin H. D) MALDI-TOF MS analysis of the reaction mixture after irradiation confirms the presence of the cyclic peptide ($[M+H]^+_{obs}$ = 611.292 Da, $[M+H]^+_{calc}$ = 611.294 Da) and reveals some free linear peptide ($[M+H]^+_{obs}$ = 629.304 Da, $[M+H]^+_{calc}$ = 629.304 Da).

ONBY75-intein precursor with the linear segetalin H sequence could be expressed in and purified from *E. coli* cells in stable form. Again, irradiation for four minutes (366 nm) resulted in intein conversion, albeit with somewhat lower conversions of approximately 20–30% (Figure 3B). Mass spectrometric analysis confirmed the presence of the cyclic pentamer; however, also the linear form of segetalin H could be detected (Figure 3D). Together, these findings show that stable precursors for peptides of various lengths can be isolated and converted into the cyclic products. Subsequent purification of the cyclic peptide from the defined splicing mixture will be clearly facilitated compared to a complex whole-cell extract. Not surprisingly, these two examples also indicate that different sequences will not be equally well suited for cyclization, potentially owing to ring strain or amino acid dependencies of the intein, which is consistent with previous observations for the SICLOPPS approach.

Interestingly, Phe75 is in close proximity to the highly conserved His73 of the blockB/N3 motif, which is of crucial

catalytic importance for the initial N→S acyl shift.[26] The crystal structure of the wild-type intein[23a] also reveals contacts to Ile150 and Val152 of the last β-strand, which contains the C-terminal splice junction at Asn154. These observations may explain why the perturbation with ONBY at position 75 gave rise to an inactive intein that is also not prone to cleavage side reactions, only when enforced by high nucleophile concentrations (Figure S4). A sequence analysis of all regular class I inteins shows the high preference for hydrophobic amino acids at this position, with Phe being the most frequently found residue.[27] Some of the well established inteins, such as the highly active naturally split *Npu* DnaE, Gp41-1, and AceL–TerL inteins,[28] indeed have a Phe at this position (Figure S7), suggesting the potential for an exciting extension of the approach described herein.

In summary, a novel light-dependent intein, the ONBY75-intein, was designed. It allows stable precursor purification for both regular splicing and for cyclic peptide formation. This is the first time that a split intein precursor for a cyclic polypeptide could be purified. Our approach facilitates the straightforward isolation of cyclic peptides in a defined mixture that additionally only contains the remaining intein components. This will be useful for the rapid production and testing of individual cyclic peptides. Furthermore, it will provide a basis for inhibitor discovery from large sequence libraries by high-throughput in vitro screening using conventional protein assays. In contrast to an uncontrolled intein reaction, our approach can circumvent potentially perturbing effects of other cellular components and potential in vivo toxicity of the cyclic peptide products. Finally, we expect the ONBY75-intein to be useful for in vivo applications by intracellular control of peptide and protein activity.

Experimental Section

Incorporation of the unnatural amino acid ONBY (added at 1 mM to the LB medium of *E. coli* BL21/DE3 cultures) was achieved by co-expression of the designated tRNA and tRNA synthetase.[21a] Protein purification was performed by Ni-NTA chromatography. UV light was administered by a standard, hand-held UV lamp (6 W). Peptide products were analyzed by MALDI-TOF MS, following a desalting step by a C18 ZipTip column of the irradiated, purified intein precursor protein.

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