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Article

A Simple Two-step System to Produce a Cyclic Peptide Library for Cell-based Assays

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Abstract: The pharmaceutical market consists mainly of chemical and biological drugs. These drugs act on different types of targets and have distinct pharmacological properties. Generally, chemical drugs bind to the active site of target enzymes and easily penetrate the cell membrane owing to their small size; however, biological drugs can bind to the protein–protein interaction site but are less stable due to their protein properties. Cyclic peptides possess the pharmacological merits of both chemical and biological drugs, such as the ability to bind to the protein–protein interaction site and penetrate cell membranes. In this study, we developed a simple two-step system to generate a cyclic peptide library using the split intein of Npu DnaE and Gateway cloning. The first step is the PCR of Ready-to-use(R) template DNA having the coding sequences of random cyclic peptides between two split intein elements NpuC and NpuN and the recombination recognition site of Gateway cloning. The second step is the transformation of the PCR products via Gateway cloning to produce colonies with expression vectors to produce cyclic peptides comprising random amino acid sequences. The expression vectors in randomly chosen transformed colonies were confirmed to have random cyclic peptide sequences and all the clones, except ones having a stop codon in the cyclic peptide coding region, showed the expected protein splicing result. This simple two-step system for bacterial expression systems may be modified to suit various expression systems for cell-based assays.

Keywords: Split-intein circular ligation of peptides and proteins (SICLOPPS); cyclic peptide libraries; protein–protein interactions; drug; gateway cloning

1. Introduction

Chemical compound libraries for high throughput screening are important for drug discovery. Numerous chemical libraries originate from diverse sources of natural and synthesized compounds, and tremendous efforts are underway to identify novel druggable materials with novel structures and pharmacological properties (Liu, Li et al. 2017). In the mid-1980s, combinatorial chemistry provided a systematic way to generate a large array of structurally diverse compounds by crosslinking various building blocks (Geysen, Meloen et al. 1984, Houghten 1985). Peptide libraries have been successfully developed based on the principles of combinatorial chemistry. Peptides show specificity and high affinity for target proteins; however, they exhibit low stability and poor cell permeability in vivo (Laraia, McKenzie et al. 2015).

Cyclic peptides, especially head-to-tail cyclic peptides, consist of the same amino acid building blocks as those in proteins; however, they display improved pharmacochemical properties in half-life stability, conformational rigidity, and cell permeability owing to the canonical peptide bond between the N-terminal and C-terminal ends (Jing and Jin 2020). Drugs with a cyclic peptide scaffold include the hormone analogs of oxytocin, octreotide, and vasopressin; the antibiotics vancomycin, daptomycin, and polymyxin B; and the immunosuppressant cyclosporine (Craik, Fairlie et al. 2013). Recently, cyclic peptides from

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actinomycetes were found to be novel anti-tuberculosis drug candidates against multi-drug resistant *Mycobacterium tuberculosis* targeting ClpC1 (Choules, Wolf et al. 2019, Wolf, Lee et al. 2019).

Cyclic peptides have a large surface area that provides a site for selective interaction with the protein targets and often have little to no toxicity owing to their benign amino acid make-up (Zorzi, Deyle et al. 2017). Further, they are resistant to hydrolysis by exo-peptidase due to the lack of both amino and carboxyl termini, and even endopeptidases, as the structure is less flexible than linear peptides (Joo 2012), which makes them more stable and durable.

Intein (*in*tervening pro*tein*), a protein that performs protein splicing in an auto-processing manner, can be used to produce cyclic peptides and cyclic peptide libraries (Nanda, Nasker et al. 2020). This protein exists in precursor polypeptides containing an intein sandwiched by two extein (*ex*ternal pro*tein*) sequences, excises itself, and ligates the two extein sequences by forming a new peptide bond via a post-translational modification process (Paulus 1998, Mills, Johnson et al. 2014, Shah and Muir 2014).

The split intein proteins, in which the N- and C-intein regions are not covalently linked to each other, can be rearranged to create cyclic peptides. The permutation of N- and C-intein elements with the target amino acid sequence between them leads to the head-to-tail cyclization of the target sequence (Scott, Abel-Santos et al. 2001, Tavassoli and Benkovic 2007, Townend and Tavassoli 2016). The split intein circular ligation of peptides and proteins (SICLOPPS) method was developed to use split inteins to produce cyclic peptides and proteins (Scott, Abel-Santos et al. 1999), offering a biosynthetic technology for generating cyclic peptide libraries. *Synechocystis* sp. PCC6803 (*Ssp*) was the first intein utilized in generating cyclic peptide libraries (Wu, Hu et al. 1998). Recently, the DnaE split intein of *Nostoc punctiforme* (*Npu*) from the α subunit of DNA polymerase III (DnaE) was shown to have a faster splicing activity and higher tolerance to extein sequence variations than *Ssp* inteins (Townend and Tavassoli 2016).

The standard restriction and ligation-based cloning is widely used for constructing protein expression vectors based on the availability of highly specific restriction enzymes. However, this cloning method can be lengthy, with relatively low success rates. In addition, the cloning of genes containing internal restriction sites that are also present in a multiple cloning site or changing expression systems is complicated (Celie, Parret et al. 2016).

The recombination-based cloning technology called "Gateway cloning" is based on the highly specific integration and excision reaction of bacteriophage λ into and out of the *Escherichia coli* genome (Hartley, Temple et al. 2000, Landy 2015). In the λ recombination pathway, the recombining DNA contains a pair of inverted repeat recombinase-binding sites that flank an identical 7 bp overlap region in both DNAs. These overlapping regions create a four-way DNA Holliday junction that can be cleaved and rearranged into recombinant products (Landy 2015). Integrative recombination between *att*P (on the phage chromosome) and *att*B (on the bacterial chromosome) is performed by integrase and integrase host factor. The excisive recombination between *att*L and *att*R sites is carried out by integrase, integrase host factor, and excisionase (Hartley, Temple et al. 2000, Landy 2015). The Gateway cloning system (Invitrogen) uses the *att* sites as specific recombination sites, and the direction of the recombination reactions can be controlled by providing different combinations of proteins and sites. In this study, the Gateway cloning method was used to attain the highly efficient cloning and transformation to produce many colonies to generate cyclic peptides.

2. Results

2.1. A simple two-step system to produce a cyclic peptide library

The previously published protocol to manufacture SICLOPPS libraries uses four sequential cloning steps (Tavassoli and Benkovic 2007). In the present study, we constructed a R(ready-to-use)-template DNA containing the two elements of C-terminal split intein (NpuC) and N-terminal split intein (NpuN) for protein splicing, nucleotide sequence for

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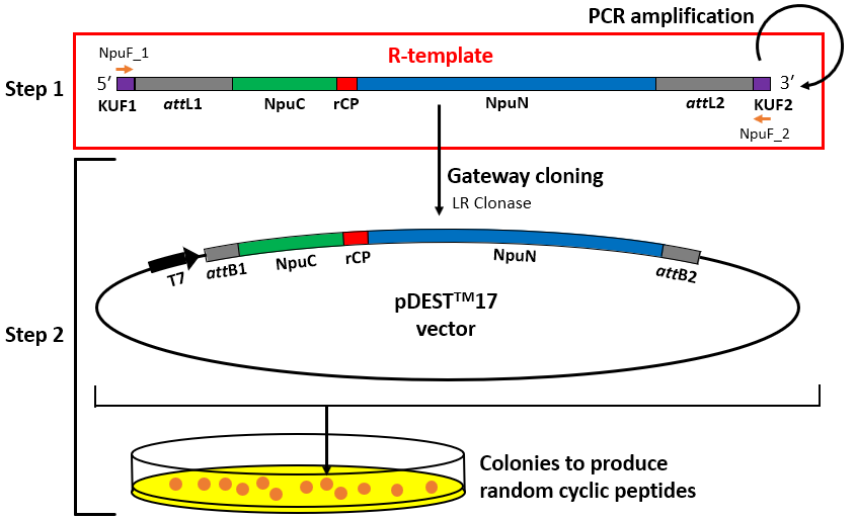
a random cyclic peptide (rCP), and recombination recognition sites at both 5′- and 3′-ends for Gateway cloning (Scheme 1). To generate random cyclic peptide expressing vectors, the R-template can be amplified by simple PCR. The resulting PCR products can be used directly for Gateway cloning to produce rCP-producing colonies.

The split intein of Npu DnaE comprises a 36-amino acid NpuC and a 102-amino acid NpuN. The NpuC and NpuN elements are expressed as the functional pre-splicing form of NpuC-rCP-NpuN (core R-template) in cells. To clone the core R-template having the protein coding region into the expression vector in Gateway cloning, the recombination recognition sites of *att*L1 and *att*L2, including the external arm sequence for LR clonase, are attached at both 5′- and 3′-ends in R-template (Figure 1).



Scheme 1. A simple two-step procedure to generate a cyclic peptide library. Step 1 is to amplify the ready-to-use template (R-template) via PCR without losing the diversity of random cyclic peptides (rCPs). Step 2 is to clone the split intein gene with a random cyclic peptide from the PCR products into the bacterial expression vectors using LR clonase in Gateway cloning. The specific cyclic peptide is spontaneously expressed from the corresponding expression vector in the transformed cells.

2.2. Construction of R-template

The NpuC and NpuN domains were amplified from the synthesized *Npu dnaE* gene via PCR (Figure S1). Resulting two main DNA fragments NpuC-rCP and NpuN were combined to form the core R-template. The PCR product of the core R-template was cloned into the pDONR™221 vector (Invitrogen) via Gateway cloning. The nucleotide sequence of core R-template in the pDONR™221 vector was verified to have no mutation in the protein coding region.

The core R-template was further modified into R-template to have both rCP between NpuC and NpuN and full recombination recognition sites for Gateway cloning in PCR products. The DNA fragment encoding a cyclic peptide of 7-mer CXXXXXX (X is a random amino acid) was inserted between NpuC and NpuN elements with the primer NpuC_j_18.

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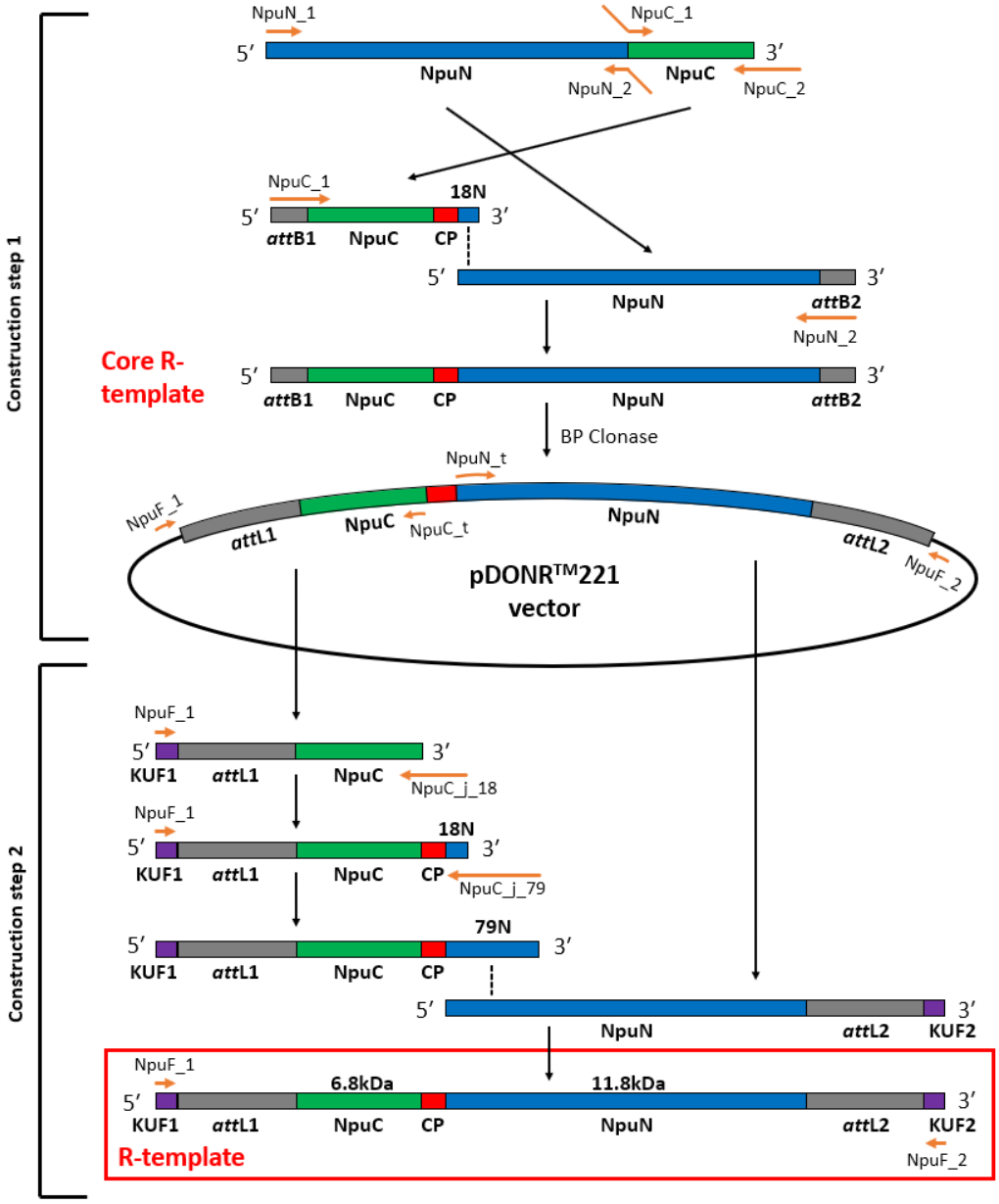

Figure 1. The overall construction scheme of the ready-to-use template (R-template). Step 1 of the construction is to produce the core ready-to-use template (core R-template) in the pDONR™221 vector. The synthesized *dnaE* gene from *Nostoc punctiforme* PCC73102 is divided into the NpuC and NpuN elements, and the cyclic peptide coding element (rCP) is inserted between the NpuC and NpuN elements. The core R-template is then inserted into the pDONR™221 vector via Gateway cloning. Step 2 of the construction is to produce the R-template via inserting rCP into between the NpuC and NpuN elements and adding the recombination recognition sites of KUF1-*att*L1 and *att*L2-KUF2 at both 5′- and 3′-ends for Gateway cloning.

The NpuC_j_18 primer has 18 degenerated nucleotide sequences encoding six random amino acids after TGC encoding cysteine (Table S1). The NpuC_j_18 primer also has overlapping 18 nucleotides with the 5′-end of NpuN, which was further extended to 79 nucleotides with the primer NpuC_j_79. Two main DNA fragments containing NpuC-rCP and NpuN were extended to have the R-template. Initially, the combining step of PCR extension was not successful and produced inaccurate PCR products. Various overlapping regions and lengths of PCR primers in the 5′-end of NpuN region were tried, and the primers NpuC_j_18 and NpuC_j_79 produced the best result of high yield and accuracy of PCR products in the PCR reactions.

In Gateway cloning, LR clonase works the cloning of DNA insert into the expression vector such as pDEST™17 (Invitrogen) via DNA recombination. The LR clonase requires the recombination recognition sites of *att*L with extra arm sequences at both 5′- and 3′-

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ends of the insert DNA fragment. The full recombination recognition sites including *att*L1 and *att*L2 with extra arm sequences are added into the core R-template with the primers NpuF_1 and NpuF_2 from the pDONR™221 vector to produce the R-template. The resulting R-template can be amplified by a simple PCR, which can be directly cloned into the pDEST™17 vector by LR clonase in the Gateway cloning.

2.3. Transformation efficiency via Gateway cloning

The solution of the PCR amplification reaction of the R-template was used for Gateway cloning. PCR solution (0.1 μL) produces approximately 30 colonies per plate, implying that a 20 μL of PCR reaction produces 6,000 rCPs in colonies.

2.4. Verification of cloned expression vectors for rCP

To confirm the random sequences of cyclic peptides in the cloned expression vectors, 12 colonies were randomly chosen from the transformed colonies, from which the expression vectors were obtain and sequenced. The sequencing results show that all the 12 cyclic peptides were nonidentical (Figure S2). There have been a limited number of mutations in the NpuC and NpuN elements in repeated experiments. In the current batch experiment, only a mutation is found in colony 3.

2.5. Protein splicing efficiency of rCP expressing Npu proteins

Protein splicing efficiency was tested for randomly chosen nine clones. Split intein Npu was expressed from the clones. The expressed Npu proteins were purified via Ni-NTA affinity purification from the N-terminal 6xHis tag attached to the NpuC element. All the nine clones showed two spliced NpuC and NpuN proteins and the pre-spliced form of full-length NpuC-rCP-NpuN proteins (Figure 2), which implies most of the expression clones produce the functional form of split intein proteins.

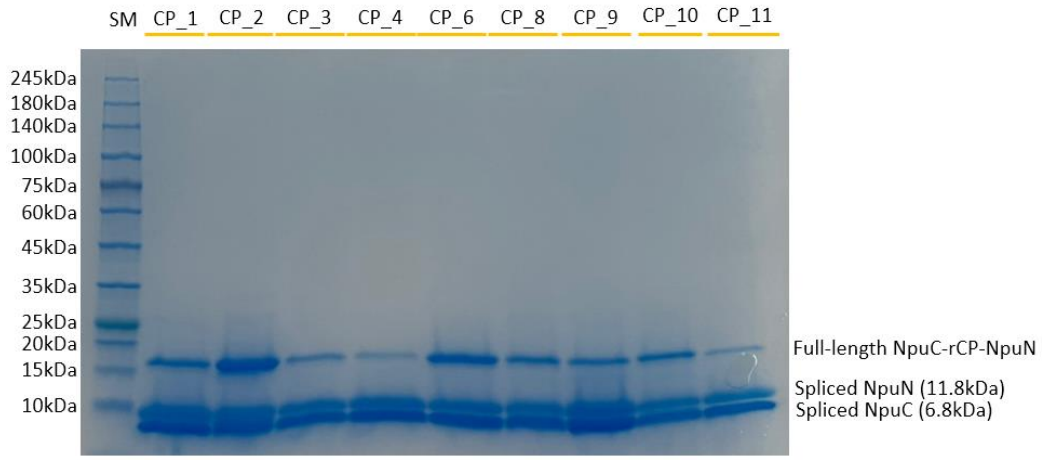

Figure 2. The protein splicing of expressed split intein Npu producing a cyclic peptide. The SDS-PAGE (Mini-PROTEAN TGX Gel of 4-20% gradient, BioRad) of purified split intein Npu is shown from randomly chosen nine clones out of 12 DNA sequence verified clones. The split intein Npu elements are purified via the Ni-NTA affinity chromatography, which shows the pre-splicing form of the full length of split intein Npu with rCP and the two spliced elements of NpuN and NpuC.

3. Discussion

The successful use of SICLOPPS via *Npu* to produce cyclic peptide libraries has been reported in previous studies (Townend and Tavassoli 2016, Kick, Harteis et al. 2017). In the method of cyclic peptide library construction described by Tavassoli et al. (Tavassoli and Benkovic 2007), cyclic peptide-expressing plasmids need to go through a handful of ligation–cloning steps to insert rCP between NpuC and NpuN elements. In the present study, we produced the R-template DNA fragment having the functional split intein Npu DnaE to produce rCP, which is ready-to-use for cell-based assays via Gateway cloning.

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The simple two-step system simplifies the cloning step and maximizes transformation efficiency to produce a cyclic peptide library.

The conventional Gateway cloning inserts the DNA fragment from PCR products into the donor vector (non-expression vector) and the insert in the donor vector is transferred to the destination vector (expression vector). We simplified the Gateway cloning via inserting the DNA fragment of NpuC-rCP-NpuN directly into a pDEST™17 expression vector. LR clonase in Gateway cloning performs the recombination reaction to produce the functional rCP expression vectors. Expressed functional split intein proteins spontaneously produced rCPs in the transformed cells, which could enable us to use the cyclic peptide library in cell-based assays. The current expression vector system could be modified for eukaryotic expression system, which will increase the possible application spectrum.

The newly developed method to produce rCP is as simple as the two-step of PCR and transformation reactions. The simplicity and ease of the rCP production system will facilitate the use of cyclic peptides for diverse screenings in drug discovery.

4. Materials and Methods

4.1. Materials

The *dnaE* gene from *N. punctiforme* PCC73102 (accession ID: CP001037.1) was synthesized with codon optimization by Bioneer Company (Korea). AccuPower® HotStart Pfu PCR PreMix (Bioneer) containing Pfu DNA polymerase was used for running PCR reactions. Each PCR product was run on a 1.5% agarose gel with 1× *Safe-Pinky* DNA Gel Staining Solution (GenDEPOT), cut to a precise size, and purified by gel-extracting purification (AccuPrep PCR/Gel Purification Kit, Bioneer).

4.2. Construction of the core R-template having functional split intein elements and rCP

The core R-template encodes the functional pre-splicing form (NpuC-rCP-NpuN) containing two split intein elements and a cyclic peptide sequence in the middle. The NpuC-rCP and NpuN DNA fragments were amplified by PCR using the primers NpuC_1 and NpuC_2 and NpuN_1 and NpuN_2, respectively (Figure S1a and S1b and Table S1). The random amino acid sequences of cyclic peptides are encoded in the NpuC_2 primer with random nucleotides of N. The resulting two main DNA fragments were combined into the core R-template via the overlap extension PCR with NpuC_1 and NpuN_2 primers (Figure S1c and Table S2). The core R-template was cloned into the pDONR™221 vector using BP clonase in Gateway cloning.

4.3. Construction of the R-template

The core R-template in the pDONR™221 vector was used to generate the R-template. The KUF1-*att*L1-NpuC and NpuN-*att*L2-KUF2 DNA fragments were amplified via PCR using the primers NpuF_1 and NpuC_t and NpuN_t and NpuF_2, respectively (Figure S1d and S1e). The rCP-encoding sequence was added to the KUF1-*att*L1-NpuC DNA fragment via PCR using NpuF_1 and Npu_j_18 (Figure S1f), of which the 3′-overlapping 18 nucleotide sequence to the 5′-NpuN sequence was further extended to 79 nucleotide sequences via PCR using the primers NpuF_1 and Npu_j_79 (Figure S1g). The resulting two main DNA fragments KUF1-*att*L1-NpuC-rCP-79N and NpuN-*att*L2-KUF2 were combined into the R-template DNA via the overlap extension PCR without a primer (Figure S1h). The R-template was amplified via PCR using NpuF_1 and NpuF_2 (Figure S1i) and cloned into the *att*R-containing destination vector pDEST™17 through a recombination reaction using LR clonase (Invitrogen) in Gateway cloning to create the *att*B-containing cyclic peptide expression-ready vectors. The cloning mixture was then transformed into DH5α and BL21(DE3) competent cells (Real Biotech Corporation) for colony formation.

4.4. Verification of DNA sequence of R-template in cloned expression vectors

The transformed DH5α cells were grown on Luria-Bertani agar medium containing 100 μg mL^{-1} ampicillin at 37°C for 12 h, and 12 colonies were randomly picked for plasmid

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extraction using the FavorPrep Plasmid Extraction Mini Kit (Favorgen Biotech). The plasmids were sent for DNA sequencing (Macrogen, Korea) for sequence validation.

4.5. Expression and purification of spliced split intein Npu DnaE elements

The rCP expression plasmids having the R-template in pDEST™17 vectors were transformed into BL21(DE3) cells. The cells were incubated at 37°C in 100mL Luria-Bertani medium containing 50 μg ml^{-1} ampicilin while shaking and induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside when the OD_{600} value reached 0.6. The induced cells were then cultured for an additional 8 h. Cell pellets were harvested by centrifugation for 30 min at 3000 × g using CRYSTE VARISPIN 12R multi centrifuge (Korea) and dissolved in the ice-cold lysis buffer containing 25 mM Tris pH 7.5, 300 mM NaCl, 30 mM imidazole, 10% glycerol, and 3 mM β-mercaptoethanol. Subsequently, cell walls were broken via sonication using sonosmasher ULH-700S (Korea) with a 20 kHz, and the crude cell extract was centrifuged for 1 hour at 21,000 x g using CRYSTE PURISPIN 17R micro centrifuge (Korea) at 4°C to obtain the supernatant containing soluble intein fragments. The supernatant was purified by Ni-NTA affinity chromatography using an open column (BIO-RAD) previously equilibrated with the lysis buffer. The intein fragments were eluted with the elution buffer containing 25 mM Tris pH 7.5, 300 mM NaCl, 250 mM imidazole, 10% glycerol, and 3 mM β-mercaptoethanol. The presence of pre-spliced form of NpuC-rCP-NpuN proteins and spliced split intein domains NpuN and NpuC was confirmed by SDS-PAGE as shown in other papers (Scott, Abel-Santos et al. 1999, Cheriyan, Chan et al. 2014). The peptide cyclization using the system was confirmed by the tandem mass spectrometry (MS/MS) (data not shown).

5. Conclusions

A new compound library with novel chemical structures and pharmaceutical properties is a valuable tool for drug discovery. Peptide libraries have been widely used because of their abundant diversity from random amino acid sequences and their high availability from combinatorial chemical synthesis. Cyclic peptides have improved pharmaceutical properties compared to linear peptides, such as higher stability, rigidity, hydrophobicity, and cell penetration efficiency. Our simple two-step system to produce a cyclic peptide library for cell-based assays is expected to provide a new and simple way to use a valuable compound library.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/xxx/s1.

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