Protein Expression

Recombinant Expression and Phenotypic Screening of a Bioactive Cyclotide Against α-Synuclein-Induced Cytotoxicity in Baker's Yeast**

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Abstract: We report for the first time the recombinant expression of fully folded bioactive cyclotides inside live yeast cells by using intracellular protein trans-splicing in combination with a highly efficient split-intein. This approach was successfully used to produce the naturally occurring cyclotide MCoTI-I and the engineered bioactive cyclotide MCoCP4. Cyclotide MCoCP4 was shown to reduce the toxicity of human α-synuclein in live yeast cells. Cyclotide MCoCP4 was selected by phenotypic screening from cells transformed with a mixture of plasmids encoding MCoCP4 and inactive cyclotide MCoTI-I in a ratio of $1:5 \times 10^4$. This demonstrates the potential for using yeast to perform phenotypic screening of genetically encoded cyclotide-based libraries in eukaryotic cells.

Cyclotides are fascinating microproteins (≈ 30 residues long) present in plants from the *Violaceae*, *Rubiaceae*, *Cucurbitaceae*, and more recently *Fabaceae* families.[1] They display various biological properties such as protease inhibitory, antimicrobial, insecticidal, cytotoxic, anti-HIV, and hormone-like activities.[2] They share a unique head-to-tail circular knotted topology of three disulfide bridges, with one disulfide penetrating through a macrocycle formed by the two other disulfides and interconnecting peptide backbones, forming what is called a cystine knot topology (Figure 1 A). This cyclic cystine knot (CCK) framework gives the cyclotides exceptional rigidity,[3] resistance to thermal and chemical denaturation, and enzymatic stability against degradation.[2] Interestingly, some cyclotides have been shown to be orally bioavailable,[4] and other cyclotides have been shown to cross
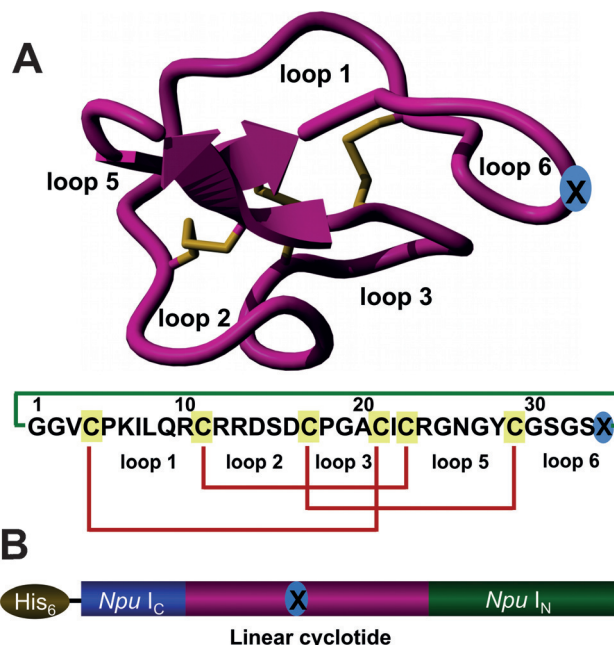
Figure 1. A) Tertiary structure of the cyclotide MCoTI-II (PDB code: 1IB9)[31] and primary structures of the cyclotides used in this work, MCoTI-I (X = D) and MCoCP4 (X = SLATWAVG). The CP4-derived peptide was grafted onto loop 6, marked with a blue circled X. The backbone-cyclized peptide (connecting bond shown in green) is stabilized by the three disulfide bonds (shown in red). B) Intein precursors used for the expression of cyclotides MCoTI-I (1a, X = D) and MCoCP4 (1b, X = SLATWAVG) in yeast *S. cerevisiae* using PTS.

the cell membrane through macropinocytosis.[5] Recent reports have also shown that engineered cyclotides can be efficiently used to target extracellular[6] and intracellular[7] protein–protein interactions. All of these features make cyclotides ideal tools for drug development to selectively target protein–protein interactions.[8]

Naturally occurring cyclotides are ribosomally produced in plants from precursor proteins[1b] and believed to be processed by specific proteases.[9] More than 200 different cyclotide sequences have been reported in the literature to date,[10] and it has been estimated by genomic analysis that about 50000 cyclotides may exist.[11] All naturally occurring cyclotides share the same CCK motif despite sequence diversity found in the loops decorating the cystine knot. Consequently, cyclotides can be considered as natural combinatorial peptide libraries structurally constrained by the cystine-knot scaffold and head-to-tail cyclization but in which hypermutation of essentially all residues is permitted with the exception of the strictly conserved cysteines that comprise the

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knot.[12] Cyclotides can be chemically synthesized, thereby permitting the introduction of specific chemical modifications or biophysical probes.[13] More recently, cyclotides have also been biosynthesized in plant-derived cell cultures[14] and prokaryotic expression cells by making use of modified protein splicing units.[15] Cyclotides have been also shown to cross cellular membranes to target intracellular protein–protein interactions.[7] Altogether, these characteristics make cyclotides ideal substrates for in-cell molecular evolution strategies to enable generation and selection of compounds with optimal binding and inhibitory characteristics.

In-cell screening and selection methods of genetically encoded cyclotide libraries provide several advantages over in vitro techniques: it ensures that hits are nontoxic, can bind the target in the appropriate cellular environment, are not rapidly degraded inside the cell, and possess high selectivity to work in living cells. In addition, this method also enables phenotypic screening for the rapid selection of novel bioactive compounds.

The use of an adequate microorganism that allows the production of large genetically encoded libraries is key for the phenotypic screening of these type of libraries. The baker's yeast *Saccharomyces cerevisiae* has been used for decades as a versatile and robust model system for eukaryotic cellular biology.[16] For example, many proteins important in human biology, including cell cycle proteins, signaling proteins, and protein-processing enzymes, were first discovered by studying their homologs in yeast.[17] In addition, several human pathologies derived from protein misfolding have been successfully modeled in simple eukaryotic organisms such as yeast *Saccharomyces cerevisiae*.[18]

To test the feasibility of expressing folded cyclotides in *S. cerevisiae*, we used the cyclotide MCoTI-I (Figure 1A). This cyclotide is a potent trypsin inhibitor ($K_i \approx 20$ pM)[19] that is naturally found in the dormant seeds of *Momordica cochinchinensis*, a plant member of the *Cucurbitaceae* family.[20] Trypsin inhibitor cyclotides are interesting candidates for drug design because they show very low toxicities to mammalian cells and can be used as natural scaffolds to generate novel biological activities.[6,7,13b,21]

To express cyclotide MCoTI-I inside living yeast cells we made use of protein trans-splicing (PTS) to facilitate the intracellular backbone cyclization (Figure 2). This process has been previously used to express small cyclic peptides[22] and more recently cyclotides[15a] in bacterial expression systems but never used before in a eukaryotic expression system to express large disulfide-containing cyclic proteins such as cyclotides. PTS-mediated backbone cyclization can be accomplished by rearranging the order of the intein fragments, that is, by fusing the I_N and I_C fragments to the C- and N-termini of the linear polypeptide precursor to be cyclized (Figure 1B). To boost the intracellular expression of folded cyclotide MCoTI-I in yeast we used the *Nostoc puntiforme* PCC73102 (*Npu*) DnaE split-intein. This DnaE intein has the highest reported rate of protein trans-splicing ($\tau_{1/2} \approx 60$ s) and has a high splicing yield.[23]

Accordingly, we designed the split-intein construct **1a** (Figure 1B), in which the MCoTI-I linear precursor was fused directly to the N- and C-termini of the *Npu* DnaE I_C and I_N
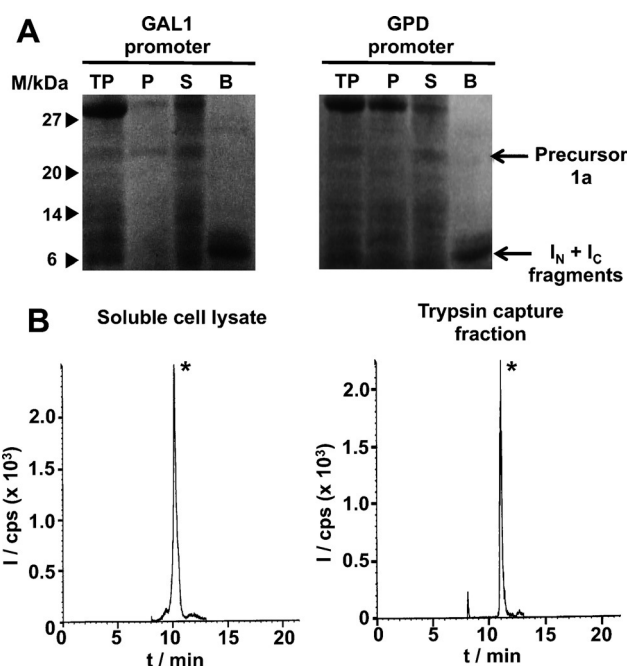
Figure 2. In-cell expression of MCoTI-I-based cyclotides in *S. cerevisiae* cells using *Npu* DnaE intein-mediated PTS. A) SDS-PAGE analysis of the recombinant expression of cyclotide precursors **1a** using a high-copy μ episomal plasmid under the control of the GAL1 (inducible, left) or GPD (constitutive, right) promoters (M stands for protein markers, TP for total cell lysate, P and S for insoluble and soluble cell lysate fractions, and B for the fraction captured by Ni-NTA-agarose beads). B) Analytical HPLC-MS/MS traces of the soluble cell extract (left) or trypsin pull-down (right) fraction from *S. cerevisiae* cells expressing precursor **1a** under the control of a GAL1 inducible promoter. The peak marked with an asterisk corresponds to folded cyclotide MCoTI-I. MS/MS analysis was performed using the ion ($m/z = 871.6$) for both Q1 and Q3 (I and cps stand for intensity and ion counts per second, respectively).

polypeptides, respectively. To facilitate backbone cyclization we used the native Cys residue located at the N-terminal position of loop 6 (Figure 1). A His-tag was also added at the N-terminus of the construct to facilitate identification of the precursor and intein-containing byproducts of the cellular cyclization process.

Expression in yeast *S. cerevisiae* of cyclotide MCoTI-I using PTS was tested by employing two different high-copy 2μ episomal expression plasmids, pYES2/NT (under the control of a GAL1 inducible promoter) and p426GPD (under the control of a GPD constitutive promoter). Expression plasmids encoding the split-intein precursor **1a** derived from plasmids pYES2/NT and p426GPD were transformed into *S. cerevisiae* strains INVSc1 and W303-1a, respectively, by electroporation. Expression of the MCoTI-precursor split-intein was accomplished for 48 h at 30 °C in media containing either 2% galactose (inducible GAL1 promoter) or 2% glucose (constitutive GPD promoter). Under these conditions the precursor was expressed at relatively high levels in both cases, ≈ 10 mg L^{-1} (GAL1 promoter) and ≈ 7 mg L^{-1} (GPD promoter). In both cases the precursor was completely cleaved (Figure 2A), indicating the intrinsic high reactivity of the split-intein construct to undergo protein trans-splicing.

Next, we quantified the amount of natively folded MCoTI-I generated in-cell by LC-MS analysis using pure MCoTI-I as standard. Correctly folded MCoTI-cyclotides are able to bind trypsin with high affinity ($K_i \approx 20$–30 pM). Therefore, this step can be used for affinity purification and to test the biological activity of the recombinant cyclotides. By either using the whole cell lysate or the fraction purified with trypsin-immobilized sepharose beads the LC-MS analysis revealed in both fractions the presence of a major peak that had the expected mass of the natively folded MCoTI-I cyclotide (Figures 2B and S1). Quantification of the amount of cyclotide gave similar yields in both fractions ca. $50 \mu g L^{-1}$ (GAL1 promoter) and $60 \mu g L^{-1}$ (GPD promoter), which correspond approximately to an intracellular concentration of approximately 450 nM and 660 nM, respectively. These results indicate that in-cell-produced cyclotide MCoTI-I is biologically active and therefore adopts a native cyclotide fold.

Encouraged by these results, we decided to express a bioactive cyclotide that could be used to perform phenotypic screening in yeast. S. cerevisiae has been shown to be a good model for many human diseases involving protein misfolding such as Parkinson's disease.[18,24] The human protein α-synuclein (α-syn) is a small lipid-binding protein that is prone to misfolding and aggregation that has been linked to Parkinson's disease by genetic evidence and its abundance in the Parkinson's disease-associated intracellular aggregates known as Lewy bodies.[25] Overexpression of human α-syn in yeast S. cerevisiae leads to endoplasmatic reticulum stress, disruption of endoplasmic reticulum–Golgi vesicle trafficking, accumulation of lipid droplets, mitochondrial dysfunction, and ultimately cell death.[25,26] This cellular pathology mirrors many aspects of the dysfunction seen in neurons and glia cells of patients with Parkinson's disease.[27] In addition, genetic screening using a yeast synucleopathy model has produced inhibitors of α-syn cytotoxicy that also resulted effective in neuronal models.[24,28]

To engineer the cyclotide MCoTI-I to inhibit α-syn-induced cytotoxicity in yeast S. cerevisiae, we used the sequence of cyclic peptide CP4 (cyclo-CLATWAVG), which was recently shown to reduce α-syn-induced cytotoxicity in a yeast synucleopathy model.[24] A CP4-derived linear peptide, in which the Cys residue was replaced by Ser, was grafted onto the cyclotide scaffold using loop 6 to provide cyclotide MCoCP4 (Figure 1). We have shown that loop 6 has the higher backbone mobility of the MCoTI-I cyclotide[3] and accordingly this loop has been shown to tolerate well the grafting of bioactive peptides.[6a,7] Replacement of the Cys residue was done to facilitate the folding of MCoCP4. Cyclotide MCoCP4 was expressed using a high-copy 2μ plasmid under the control of a GPD constitutive promoter as described above for cyclotide MCoTI-I. Under these conditions, the MCoCP4 split-intein precursor 1b (Figure 1B) was expressed with a yield similar to that of the MCoTI-I precursor (≈ 9 mg L^{-1}). As before, the precursor was completely processed in vivo (Figure 3A). LC-MS analysis of the soluble cell lysate revealed the presence of a major peak with the expected mass for the natively folded MCoCP4 cyclotide (Figures 3B and S3). Quantitative LC-MS analysis of the cell lysate provided an expression yield of 45 μg L^{-1}, which
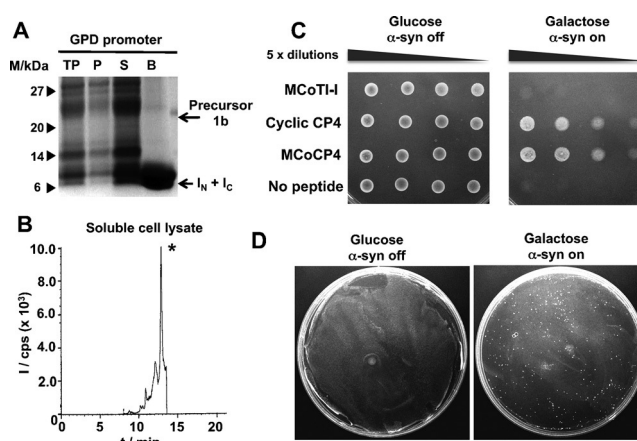
Figure 3. Expression of cyclotide MCoCP4 in S. cerevisiae cells using *Npu* DnaE intein-mediated PTS. A) SDS-PAGE analysis of the recombinant expression of cyclotide precursor 1b in S. cerevisiae strain W303-1a cells for in-cell production of cyclotide MCoCP4 (lane description as in Figure 2). B) Analytical HPLC-MS/MS trace of the soluble cell extract fraction from S. cerevisiae cells expressing precursor 1b under the control of a GPD constitutive promoter. The peak marked with an asterisk corresponds to folded cyclotide MCoCP4. MS/MS analysis was performed using the ion ($m/z = 1038.8$) for both Q1 and Q3 (units are as described in Figure 2B). C) Spotting assays of normalized, serially diluted cells of the synucleopathy model expressing cyclotides MCoTI-I (negative control) and MCoCP4, and cyclic peptide CP4 (positive control). D) Phenotypic screening using a yeast synucleopathy model. Screening cells were transformed with a mixture of p426GPD plasmids encoding cyclotides MCoCP4 (cytoprotective) and MCoTI-I (inactive) in a ratio of $1:5 \times 10^4$. Glucose and galactose plates are shown to illustrate how a single bioactive cyclotide can be isolated from more than 10^7 transformants on a single plate.

corresponds to an intracellular concentration of about 390 nM. To confirm the identity of the cyclotide MCoCP4 expressed in yeast, this cyclotide was chemically produced using a GSH-induced cyclization–folding one-pot reaction as previously described.[6a,7] The cyclization–folding reaction was very efficient and complete in less than 24 h (Figure S6). Pure synthetic cyclotide MCoCP4 was shown to co-elute by HPLC-MS with yeast-produced MCoCP4 and adopt a native cyclotide fold by NMR (Table S2 and Figures S7 and S9).

To test the cytoprotective activity of cyclotide MCoCP4 we used a yeast synucleopathy model previously described in the literature.[26,29] This cellular model employs a GAL1 promoter to tightly control the expression of human α-syn encoded in the genomic DNA of the microorganism. In this way, α-syn can be expressed or totally repressed only when the yeast are grown on medium containing either galactose or glucose, respectively. Accordingly, we expressed the cyclotide MCoCP4 using a compatible μ episomal expression vector under the control of a GPD constitutive promoter using the yeast synucleopathy model described above. The inactive cyclotide MCoTI-I and cyclic peptide CP4 were also used as negative and positive controls, respectively. After transforming the cells with the plasmids encoding the different cyclic peptides, the yeast cells were grown first in medium containing 2% raffinose as carbon source and then serially spotted in media containing either 2% glucose (α-syn off) or 2% galactose (α-syn on; Figure 3C). Expression of the corre-

sponding cyclotides under these conditions did not affect significantly their expression level. As expected, no cytotoxicity was observed when the cells were grown in the presence of 2% glucose (Figure 3C), thereby indicating the total repression of α-syn expression as well as the lack of cytotoxicity of cyclotides MCoTI-I and MCoCP4. When cells were grown in the presence of galactose, which activates α-syn expression, cells coexpressing inactive cyclotide MCoTI-I were unable to grow at all dilutions tried in the assay (Figures 3C and S8). As anticipated, coexpression of cyclic peptide CP4 was able to rescue the cytotoxic phenotype induced by α-syn therefore confirming the protective activity of this peptide.[24] Remarkably, cells expressing the engineered cyclotide MCoCP4 were also able to suppress the α-syn-induced cytotoxicity under the conditions used in this assay. The cytoprotective effect of MCoCP4 was similar to that of the peptide CP4. Preliminary results indicated that cyclic peptide CP4 can be cyclized by PTS using the *Npu* DnaE split-intein providing an intracellular concentration of ca. 5 μM as quantified by LC-MS/MS (Figure S2).

Finally, we decided to explore the feasibility of using the yeast synucleopathy model for the phenotypic screening of bioactive cyclotides against α-synuclein-induced cytotoxicity. For this purpose we generated a mixture of plasmids encoding MCoCP4 and MCoTI-I in a ratio of $1:5 \times 10^4$. This DNA mixture was transformed by electroporation to provide ca. 10^7 transformants as determined by plating on medium containing 2% glucose. To perform the phenotypic screening the whole cell-based mixture was plated on media containing 2% galactose to activate the expression of α-syn and incubated for three days. Under these conditions ca. 150 colonies were obtained (Figure 3D). 30 different colonies were picked, the corresponding plasmids isolated and analyzed by DNA sequencing to confirm the identity of the encoded cyclotide. Almost all the colonies analyzed (27 of 30, 90%) provided DNA sequences encoding cyclotide MCoCP4, therefore demonstrating the feasibility of using yeast as eukaryotic platform to perform phenotypic screening of in-cell-generated bioactive cyclotides. The appearance of a small amount of false positives probably stemmed from spontaneous genomic suppressor mutations, which are common in most yeast selection schemes.[24]

In summary we have shown that bioactive folded cyclotides can be produced in eukaryotic microorganisms such as yeast *S. cerevisiae* by PTS using highly efficient split inteins. This approach was successfully used for the production of a novel cyclotide (MCoCP4) that was able to inhibit α-syn-induced cytotoxicity in live yeast cells, therefore allowing us for the first time to perform a phenotypic screen to select cyclotide sequences with biological activity from inactive cyclotides in a yeast synucleopathy model. These exciting results open the possibility to perform in-cell phenotypic screens against other cellular pathologies using cyclotide-based libraries. Using eukaryotic microorganisms for screening purposes should provide a more biologically relevant cellular background for phenotypic screening but also facilitate the production of cyclotide-based libraries containing posttranslational modifications such as phosphorylation and/or glycosylation, which are not available in bacterial expression systems. In addition, it should also allow screening cyclotides against proteins containing posttranslational modifications. Moreover, the use of the cyclotide molecular scaffold, which possesses unique pharmacological features such as extreme stability,[30] oral bioavailability,[6b] and ability to cross cellular membranes,[5] should provide an extremely valuable screening platform for the selection of novel therapeutic leads.

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