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Efficient recombinant expression of SFTI-1 in bacterial cells using intein-mediated protein trans-splicing

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Abstract

We report for the first time the recombinant expression of bioactive wild-type sunflower trypsin inhibitor 1 (SFTI-1) inside *E. coli* cells by making use of intracellular protein trans-splicing in combination with a high efficient split-intein. SFTI-1 is a small backbone-cyclized polypeptide with a single disulfide bridge and potent trypsin inhibitory activity. Recombinantly produced SFTI-1 was fully characterized by NMR and was observed to actively inhibit trypsin. The in-cell expression of SFTI-1 was very efficient reaching intracellular concentration $\approx 40 \mu\text{M}$. This study clearly demonstrates the possibility of generating genetically-encoded SFTI-based peptide libraries in live *E. coli* cells, and is a critical first step for developing in-cell screening and directed evolution technologies using the cyclic peptide SFTI-1 as a molecular scaffold.

Keywords

sunflower trypsin inhibitor; Bowman-Birk inhibitor; backbone-cyclized peptides; protein splicing; split-intein

Introduction

Sunflower trypsin inhibitor 1 (SFTI-1) is a 14 amino acid backbone-cyclized peptide containing a single disulfide bond (Fig. 1) that is naturally found in the seeds of sunflower (*Helianthus annuus*) [1]. SFTI-1 belongs to the Bowman-Birk inhibitor (BBI) family, whose members are found in many plants and are potent serine protease inhibitors [2]. The cyclic peptide SFTI-1 is the smallest and most potent BBI with a K_i against trypsin that has been reported to be in the low nanomolar range [1]. Structural analysis of SFTI-1 reveals a well-defined double β -hairpin loop linked by two short antiparallel β -strands [1, 3, 4] (Fig. 1). Previous studies have shown that SFTI-1 readily adopts its simple native fold in aqueous solution without the need of an oxidizing redox buffer system [5].

Due to its relatively rigid backbone conformation [6], the protease-binding loop is well-defined and can serve as a general scaffold for serine protease inhibitors. For example, the introduction of mutations within the protease-binding loop of SFTI-1 has been shown to produce SFTI-analogs able to inhibit other serine proteases [7–11]. SFTI-1 also possesses an exceptional stability to thermal or enzymatic degradation as a consequence of the backbone cyclization combined with the presence of an internal disulfide bond and an extensive hydrogen binding network [10, 12]. In addition, SFTI-1 has been shown to be non-toxic to mammalian cells and be able to cross cellular membranes [13]. Moreover, the SFTI-1 molecular framework can be also readily re-engineered by incorporating foreign biological active peptide sequences into one the loops to produce SFTI-analogs with novel biological activities [14–17]. All these characteristics make SFTI-1 an ideal molecular scaffold for drug development to selectively target specific protein-protein interactions [18].

In nature, SFTI-1 is ribosomally produced from a seed storage protein albumin that is processed by an endogenous specific asparaginyl endopeptidase [19]. SFTI-1 can also be chemically synthesized using an intramolecular version of native chemical ligation (NCL) [20], which allows the incorporation of specific chemical modifications or biophysical probes [21]. More recently, SFTI-1 has been biosynthesized using standard bacterial expression systems using an intramolecular version of expressed protein ligation (EPL) [22]. This method relies on the use of a modified protein splicing unit in combination with an in-cell intramolecular NCL reaction to perform the backbone cyclization of the linear peptide precursor [23, 24].

In the present work we report an alternative approach for the recombinant expression of SFTI-1 using protein trans-splicing (PTS) to facilitate the in-cell cyclization process and improve the expression yield (Fig. 2). SFTI-1 obtained by in-cell PTS was fully active and adopted a native structure as determined by NMR. In-cell expression of SFTI-1 by PTS was also highly efficient, reaching intracellular concentrations $\approx 40 \mu\text{M}$, therefore allowing this approach to be used for in-cell screening of genetically-encoded libraries based on this scaffold.

Results and discussion

In order to boost the expression of SFTI-1 in living bacterial cells we explored the use of PTS to facilitate the in-cell cyclization process and to improve the expression yield. 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, but they can bind to each other with high specificity under appropriate conditions to form an active protein splicing or intein domain in *trans* [25]. PTS-mediated backbone cyclization can be accomplished by fusing the I_N and I_C fragments to the C- and N-termini of the polypeptide for cyclization. Once the two split-intein fragments bind to each other in an intramolecular fashion, the concomitant trans-splicing reaction yields a backbone-cyclized polypeptide (Fig. 2A) [26]. This approach has been recently used for the efficient biosynthesis of cyclotides using bacterial and yeast expression systems [27, 28].

We used the *Nostoc punctiforme* PCC73102 (*Npu*) DnaE split-intein for the PTS-mediated in-cell expression of SFTI-1. This DnaE split-intein has the highest reported rate of protein trans-splicing ($\tau_{1/2} \approx 60$ s) and has a high splicing yield [29, 30]. Also, in contrast with other split-inteins, the *Npu* DnaE split-intein has been also shown to have minimal sequence requirements at the intein-extein junctions [29, 30], which allows the production of native SFTI-1 without the need to add extra extein residues to facilitate the trans-splicing reaction.

SFTI-1 is a relatively simple cyclic peptide that contains two cysteine residues that may be used for PTS. Accordingly, we designed two split-intein constructs, **1a** and **1b** (Fig. 2B). In this constructs the corresponding SFTI-1 linear precursor was fused in-frame at the C- or N-termini directly to the *Npu* DnaE I_N and I_C polypeptide, respectively. We used the native residues Cys3 (construct **1a**) and Cys11 (construct **1b**) to facilitate the PTS-mediated backbone cyclization (Fig. 2B). A His-tag was also added at the N-terminus of the constructs to facilitate identification and purification.

In-cell expression of the SFTI-1 using PTS-mediated backbone cyclization was achieved by transforming the plasmid encoding the split-precursors **1a** or **1b** into Origami 2(DE3) cells to facilitate oxidative folding. The SFTI-precursor split-intein constructs **1a** and **1b** were over expressed for 18 h at room temperature. Under these conditions the corresponding precursors were expressed at very high levels (≈ 110 mg and ≈ 130 mg for precursors **1a** and **1b**, respectively) and were completely cleaved (Fig. 3A). The extremely high reactivity of this precursor prevented us from performing a full characterization of the precursor protein including kinetic studies of the trans-splicing induced reaction *in vitro*. The extremely high reactivity of constructs **1a** and **1b**, which have both totally different extein-intein junctions, highlights the low sequence requirements of this split-intein to allow efficient trans-splicing.

Next, we tried to isolate the natively folded SFTI-1 generated in-cell by incubating the soluble fraction of a fresh cell lysate with trypsin-immobilized sepharose beads. SFTI-1 is able to bind trypsin with high affinity ($K_i \approx 0.1\text{--}1$ nM) [31, 32]. Therefore, this step can be used for affinity purification and to test the biological activity of the recombinantly-produced SFTI-1 [22]. This approach has been also successfully used for the purification of trypsin inhibitory cyclotides [27, 28, 33]. After extensive washing, the trypsin bound products were eluted with a solution containing 8 M guanidinium chloride (GdmCl) and analyzed by HPLC. The HPLC analysis revealed the presence of a major peak that had the expected mass of the native SFTI-1 independently of the construct used (Figs. 3B and 3C). It is worth noting that linear SFTI-1 ($\approx 20\%$) was also detected in the mixture after affinity purification with trypsin-sepharose beads. The presence of linear SFTI-1 was attributed to the cleavage of the peptide bond between the residues of Lys5 and Ser6. SFTI-I has been shown to reach equilibrium between the cyclic and the linear forms when incubated with trypsin [34]. In fact, this approach has been used for the production of SFTI-1 from a linear precursor containing Ser6 as N-terminal residue and using trypsin for the chemoenzymatic cyclization step [34]. To further confirm that the linear SFTI-1 was produced during the purification step, we incubated purified SFTI-1 with trypsin beads and then analyzed the absorbed components by HPLC. As expected, after incubation with trypsin an equilibrium between the cyclic ($\approx 80\%$) and linear ($\approx 20\%$) forms was rapidly reached (Fig. S1). Likewise, when the linear form isolated from the affinity purification with trypsin was

incubated with trypsin beads the same equilibrium between the cyclic and linear forms was reached (Fig. S2). It should be noted that the linear precursors that could be obtained by N- and C-terminal cleavage of the split intein polypeptides from constructs **1a** and **1b** could never be cyclized by trypsin as the N-terminal residues in these polypeptides correspond to residues Cys3 (**1a**) and Cys11 (**1b**) (Figs. 1 and 2). The SFTI-1 expression yield was estimated to be in both cases $\approx 180 \mu\text{g/L}$ of bacterial culture using LB medium, which corresponds to an intracellular concentration of $\approx 40 \mu\text{M}$ [27]. As expected, PTS-produced SFTI-1 was able to inhibit pancreatic trypsin with a K_i of $1.0 \pm 0.2 \text{ nM}$ (Fig. 4). This K_i constant closely parallels previously reported values [31, 32]. In-cell produced SFTI was also fully characterized by NMR (Fig. 5, Fig. S4 and Table S1). The protein was monodispersed and possessed a single conformation in solution. The trans configuration of Pro9 and Pro13 peptide bonds was verified by observing strong NOESY peaks between $\text{H}\alpha$ of Ile10 and $\text{H}\alpha$ of Pro9, and $\text{H}\beta$ of Phe 12 and $\text{H}\delta$ of Pro13, respectively. Pro8 was in cis configuration as evidenced by a strong NOESY peak between $\text{HN}\alpha$ of Ile10 and $\text{H}\alpha$ of Pro9. These proline conformations are in agreement with [31]. The proton assignments are also similar to those published by Craik [31] despite the fact that the latter were obtained at non physiological conditions. We concluded that in-cell produced SFTI-1 maintains the native fold.

Discussion and conclusions

We have shown that PTS-mediated backbone cyclization using the highly efficient *Npu* DnaE split-intein can be employed for the efficient production of SFTI-1 inside live *E. coli* cells. We estimate that the in-cell production of SFTI-1 was around 10 times more efficient using *Npu* DnaE PTS than EPL in combination with the *Mxe* GyrA intein [22], thereby providing an attractive alternative for the production of these types of polypeptides in bacterial cells. The use of PTS-mediated backbone cyclization has been also shown to be more efficient in the recombinant production of other backbone-cyclized polypeptides, such as cyclotides [27, 28] and θ -defensins (manuscript in preparation).

The high efficiency of PTS-mediated backbone cyclization may be explained by our choice of split-intein. The *Npu* DnaE split-intein is extremely efficient, exhibiting fast kinetics ($\tau_{1/2} \approx 60 \text{ s}$) and good yields ($\approx 80\%$) [35]. In addition, this split-intein has shown to be highly tolerant on the sequence composition at the intein-extein junctions allowing efficient protein trans-splicing and production of native backbone-cyclized polypeptides [27, 28]. Our results confirm these findings, for example constructs **1a** and **1b**, with completely different extein sequences at the extein-intein junctions (Fig. 2B), were both highly reactive in protein trans-splicing activity producing similar amounts of correctly folded SFTI-1 (Fig. 3B). In contrast, the ortholog *Ssp* DnaE intein, which has been also used for the production of small backbone-cyclized polypeptides, requires at least 4 native residues at the N- and C-terminal extein-intein junctions to work efficiently [36]. The use of split-inteins with minimal sequence requirements at the extein-intein junction is key for the production of genetically-encoded peptide libraries, where randomized polypeptide loops can be in proximity to the intein-extein junctions, and therefore could affect the PTS-mediated cyclization of some of the library peptides.

The in-cell production of bioactive backbone cyclic peptides like SFTI-1 containing a disulfide bond may have tremendous potential for drug discovery. Previous studies have shown that SFTI-1 may provide an ideal scaffold for introducing novel biological activities [7–11, 14–17] as well as for the biosynthesis of large combinatorial libraries inside of living *E. coli* cells [22]. Coupled to an appropriate in-vivo reporter system, these libraries could rapidly be screened using high throughput technologies such as fluorescence activated cell sorting [27, 37].

Materials and Methods

Analytical HPLC was performed on a HP1100 series instrument with 220 and 280 nm detection using a Vydac C18 column (5 micron, 4.6 × 150 mm) at a flow rate of 1 mL/min. Semipreparative HPLC was performed on a Waters Delta Prep system fitted with a Waters 2487 ultraviolet-visible (UV/Vis) detector using a Vydac C18 column (15–20 µm, 10 × 250 mm) at a flow rate of 5 mL/min. All runs used linear gradients of 0.1% aqueous trifluoroacetic acid (TFA, solvent A) vs. 0.1% TFA, 90% acetonitrile in H₂O (solvent B). Electrospray mass spectrometry (ESMS) analysis was performed on an Applied Biosystems API 3000 triple quadrupole electrospray mass spectrometer using Analyst 1.4.2. Calculated masses were obtained using Analyst 1.4.2. Protein samples were run on 4–20% Tris-Glycine Gels (Lonza). The gels were then stained with Pierce Gelcode Blue (Pierce), photographed/digitized using a Kodak EDAS 290, and quantified using NIH ImageJ software. DNA sequencing was performed by Retrogen DNA facility (San Diego, CA), and the sequence data were analyzed with DNASTar Lasergene v5.5.2. All chemicals were obtained from Aldrich (Milwaukee, WI) or Novabiochem (San Diego, CA) unless otherwise indicated. Restriction enzymes were purchased from New England Biolabs. Primers were ordered from IDT (Integrated DNA Technologies).

Cloning of expression plasmid encoding constructs 1a (pASK-SFTI-C3) and 1b (pASK-SFTI-C11)

The dsDNA encoding the SFTI-1 linear precursors was amplified by polymerase chain reaction (PCR) using 5′ - TCA AAA ATG GCT TCA TAG CTT CGA ACT GCA CCA AAT CTA TCC CGC CGA TCT GCT TCC CGG ACG GTC GTT GTT TAT CAT ATG AAA CGG AGA TCT TGA CAG - 3′ (construct **1a**) or 5′ - TCA AAA ATG GCT TCA TAG CTT CGA ACT GCT TCC CGG ACG GTC GTT GCA CCA AAT CTA TCC CGC CGA TCT GTT TAT CAT ATG AAA CGG AGA TCT TGA CAG - 3′ (construct **1b**) as template. The 5′-primer (5′-TCA AAA ATG GCT TCA TAG CTT CG-3′) encoded a *BstB* I restriction site. The 3′-primer (5′-CTG TCA AGA TCT CCG TTT CAT ATG AT-3′) encoded a *Bgl*II. The PCR products were digested with *BstB* I and *Bgl*II, and ligated into a *BstB* I, *Bgl*II-treated pASK-TS-MCoTI (see below) to give the plasmids pASK-SFTI-C3 (construct **1a**) and pASK-SFTI-C11 (construct **1b**).

Cloning of pASK-TS-MCoTI

The gene encoding the Npu DnaE I_C and I_N polypeptides fused to a linear precursor of cyclotide MCoTI-I was obtained by digesting plasmid pET28-TS-MCoTI [27] was digested

with *Xba*I and *Hind*III and ligated into *Xba*I, *Hind*III-treated pASK-IBA35 plasmid (Iba Life Sciences) to give pASK-TS-MCoTI.

Expression and purification of SFTI-1 in *E. coli*

E. coli Origami 2(DE3) cells (Novagen) were transformed with plasmids pASK-SFTI-C3 or pASK-SFTI-C11. Expression was carried out in 1 L of 2XYT or M9 media containing ampicillin (100 µg/L) at room temperature. Briefly, 5 mL of an overnight starter culture were used to inoculate 1 L of 2XYT or M9 media. Cells were grown to an OD at 600 nm of ≈ 0.6 at 37° C. Protein expression was induced by addition of anhydrotetracycline hydrochloride (AHT) to a final concentration of 200 µg/L at room temperature for overnight. The cells were harvested by centrifugation, resuspended in 30 mL of lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl at pH 8.0 containing 5% glycerol) and then lysed by sonication. The lysate was clarified by centrifugation at 15,000 rpm in a Sorval SS-34 rotor for 30 min. The clarified supernatant was incubated with 1 mL Ni-NTA agarose beads (Qiagen), previously equilibrated with lysis buffer at 4° C for 1 h with gentle rocking. The beads were separated from the cell lysate by centrifugation and then extensively washed with 50 bed-volumes of wash buffer (20mM imidazole, 50 mM NaH₂PO₄, 300 mM NaCl buffer at pH 8.0). Quantification of the level of expression was performed by quantifying the I_C and I_N polypeptides by SDS-PAGE analysis (Fig. 4A) was performed to quantify the expression level of intein. The expression level for intein precursor **1a** and **1b** was estimated to be ≈ 110 mg/L and 135 mg/L, respectively.

Capture of SFTI-1 using trypsin-immobilized sepharose beads

Trypsin-immobilized agarose beads were prepared as previously described [27]. Briefly, NHS-activated sepharose was washed with 15 volumes of ice-cold 1 mM HCl. Each volume of beads was incubated with an equal volume of coupling buffer (200 mM NaH₂PO₄, 250 mM NaCl buffer at pH 6) containing 2–4 mg of porcine pancreatic trypsin type IX-S (14,000 units/mg)/mL for 3 h with gentle rocking at room temperature. The beads were then rinsed with 10 volumes of coupling buffer, and incubated with excess coupling buffer containing 100 mM ethanolamine (Eastman Kodak) for 3 h with gentle rocking at room temperature. Finally, the beads were washed with 50 volumes of washing buffer (200 mM NaOAc, 250 mM NaCl buffer at pH 4.5) and stored at 4°C until use. The sepharose-trypsin beads are stable for a 6 month under these conditions. Affinity chromatography purification of SFTI-1 was carried out as followed: 30 mL of clarified lysate obtained previously was incubated with 500 µL of trypsin-sepharose for one hour at room temperature with gentle rocking, and centrifuged at 3,000 rpm for 1 min. The beads were washed with 50 volumes of column buffer (50mM NaH₂PO₄, 0.1 mM EDTA, 250 mM NaCl buffer at pH 7.2) containing 0.1% Triton and then rinsed with 50 volumes of column buffer without detergent. The sepharose beads were treated with 3 $\times$ 0.5 mL of 8 M GdmCl at room temperature for 15 min and then eluted by gravity. The eluted fractions containing SFTI-I were analyzed by HPLC and ES-MS [SFTI-1; expected averaged mass 1513.8 Da, found mass 1513.1 $\pm$ 0.1 Da, ¹⁵N-labeled SFTI-1; expected averaged mass 1531.8 Da, found mass 1531.3 $\pm$ 0.7 Da.]. SFTI-1 was purified by semipreparative HPLC using a linear gradient of 0–70% buffer B over 30 min. The expression yield for folded SFTI-1 was estimated to be ≈ 170 µg/L and ≈ 185 µg/L for constructs **1a** and **1b**, respectively

Determination of K_i

Trypsin inhibitory assay was performed in a solution containing 20 mM CaCl_2 , 50 mM Tris•HCl buffer at pH 8.0. Inhibitor solutions of SFTI-1 ranging from 1 pM nM to 100 nM were preincubated with a freshly prepared solution of 5 nM trypsin in a final reaction volume of 100 μL . After incubation for 30 minutes, residual trypsin activity was measured by adding the fluorogenic substrate $\text{N}^\alpha\text{-Cbz-L-Arg-AMC}$ (AMC: 7-amido-4-methylcoumarin, Cbz: benzyloxycarbonyl) to a final concentration of 3 μM and then following the release of AMC using a using an Envision 2103 plate reader (PerkinElmer). Measurements were taken every 30 seconds using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The initial velocities for the hydrolysis of substrate $\text{N}^\alpha\text{-Cbz-L-Arg-AMC}$ by trypsin in the presence of different concentrations of SFTI-1 were fitted to a one-site competitive binding equation using the software package Prism (GraphPad Software). K_i was calculated using the equation of Cheung and Prusoff [38] and a K_m value of 12 μM [39].

NMR spectroscopy

NMR samples were prepared by dissolving peptides into 80 mM potassium phosphate pH 6.5 in 90% $\text{H}_2\text{O}/10\% \text{ } ^2\text{H}_2\text{O}$ (v/v) to a concentration of approximately 0.2 mM for SFTI-1. All NMR spectra were acquired on Bruker Avance II 700 MHz spectrometers equipped with TXI cryoprobe. The NMR spectra were acquired at 298 K, and 2,2-dimethyl-2-silapentane-5-sulfonate, DSS, was used as an internal reference. The carrier frequency was centered on the water signal, and the solvent was suppressed by using WATERGATE pulse sequence. 3D ^1H , ^{15}N -NOESY (mixing time 200 ms) and 3D ^1H , ^{15}N -TOCSY (spin lock time 80 ms) were collected using 1024 t_3 points, 256 t_2 and 64 t_1 points in direct proton and indirect proton and nitrogen dimensions with 8 transients. 2D ^1H , ^1H -TOCSY (spin lock time 80 ms) and ^1H , ^1H -NOESY (mixing time 200 ms) spectra were collected using 4096 t_2 points and 256 t_1 of 64 transients. Spectra were processed using Topspin 1.3 (Bruker). Each 3D-data set was apodized by 90°-shifted sinebell-squared in all dimensions, and zero filled to 1024 $\times$ 256 $\times$ 64 points prior to Fourier transformation. Each 2D-data set was apodized by 90°-shifted sinebell-squared in all dimensions, and zero filled to 4096 $\times$ 512 points prior to Fourier transformation. Assignments for the backbone nitrogen (^{15}N) and protons (Ha and NH) of folded SFTI-1 (Table S1) were obtained using standard procedures [40, 41].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Primary and tertiary structure of the cyclic peptide SFTI-1 (PDB code: 1JBL) [31]. The disulfide bond and backbone cyclization are indicated with yellow and blue lines, respectively. Molecular graphics were created using Yasara (www.yasara.org).

Figure 2.

Biosynthesis of SFTI-1 in *E. coli* cells. **A.** In-cell production of natively folded SFTI-1 in bacterial cells using PTS-mediated backbone cyclization and oxidative folding. **B.** Sequences of the linear precursors used for in-cell production of SFTI-1 production of SFTI-1. The precursors contain an N-terminal containing linear precursor of SFTI-1 fused to the N- and C-termini of the Npu DnaE I_N and I_C polypeptides, respectively. The sequence corresponding to loop 1 is underlined as a reference. The His tag located at the N-terminus of the constructs is not shown for clarity.

Figure 3.

In-cell expression of SFTI-I in *E. coli* cells using *Npu* DnaE intein-mediated PTS. **A.** SDS-PAGE analysis of the recombinant expression of cyclotide precursors **1a** and **1b** in Origami2(DE3) cells for in-cell production of SFTI-1. **B.** Analytical HPLC trace of the soluble cell extract of bacterial cells expressing precursors **1a** and after purification by affinity chromatography on a trypsin-sepharose column. The presence of linear SFTI-1 ($\approx 20\%$) was attributed to trypsin cleavage during the purification process. Endogenous bacterial proteins that bind trypsin are marked with an asterisk. **C.** HPLC and ES-MS analysis of purified recombinant SFTI-1. Natively folded SFTI-1 is marked with an arrow. The expected average molecular weight is shown in parentheses.

Figure 4. Inhibition of trypsin by recombinant SFTI-1. Plot of the relative initial velocities for the hydrolysis of substrate N^α-CBZ-L-Arg-AMC by trypsin in the presence of different concentrations of SFTI-1. K_i was calculated using the following equation: $K_i = IC_{50} / (1 + S_0 / K_m)$, where S_0 is the substrate concentration and K_m is the Michaelis constant [39].

Figure 5. Structural characterization of recombinant SFTI-1 by NMR. ^{15}N -HSQC spectrum of [U - ^{15}N]-labeled SFTI-1 in aqueous 80 mM potassium phosphate buffer at pH 6.5.