

Protein Cyclization Enhanced Thermostability and Exopeptidase-Resistance of Green Fluorescent Protein

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A mutant of green fluorescent protein (GFPmut3*) from the jellyfish *Aequorea victoria* was cyclized *in vitro* and *in vivo* by the use of a naturally split intein from the *dnaE* gene of *Synechocystis* species PCC6803 (Ssp). Cyclization of GFPmut3* was confirmed by amino acid sequencing and resulted in an increased electrophoretic mobility compared with the linear GFPmut3*. The circular GFPmut3* was 5°C more thermostable than the linear form and significantly more resistant to proteolysis of exopeptidase. The circular GFPmut3* also displayed increased relative fluorescence intensity. In addition, chemical stability of GFPmut3* against GdnHCl revealed more stability of the circular form compared with the linear form.

Keywords: Intein, protein cyclization, green fluorescent protein, purification

An ideal and robust enzyme should be catalytically efficient, thermostable, and proteolysis-resistant. Unfortunately, naturally occurring enzymes often lack one or more of these properties. Many studies were undertaken with the aim of enhancing the stability of naturally occurring proteins. Directed evolution approaches such as site-directed mutagenesis [18], exchanging the active sites [15] or designing structure-based chimeric enzymes [12], and the consensus concept for thermostability engineering of proteins [14, 16] are still the common means to improve the stability of proteins.

One method, termed intein-mediated protein cyclization, could further increase the stability of proteins by cross-linking N and C termini of the target proteins [3, 10, 19]. The termini of proteins of interest should be in reasonably

close proximity in their folded structures [10, 11, 20]. Protein cyclization is of special interest since it could be used to enhance the stability of enzymes, in particular in certain industrial applications. The termini of linear proteins are often flexible and easily become the targets of proteolytic enzymes. However, circular proteins are different from linear proteins, because their termini are seamlessly stitched together by native peptide bonds that can increase their thermostability and resistance to chemical, thermal, or enzymatic degradation [10, 23]. Cyclized proteins with enhanced stability could prove to have a range of new applications as drugs and agricultural agents. Furthermore, firefly luciferase was also cyclized for real-time sensing in living mammals [13]. In addition, protein cyclization also provides a powerful tool for manipulation and the understanding of protein structure and function.

Herein, we describe the use of a naturally split intein from the *dnaE* gene of *Synechocystis* sp. PCC6803 (*Ssp* DnaE intein) [25], to cyclize a mutant of green fluorescent protein (GFPmut3*) of the jellyfish *Aequorea victoria* *in vivo* and *in vitro*. GFPmut3* has highly shifted excitation maxima and its fluorescence intensity is significantly enhanced compared with wild-type GFP [1, 2]. The cyclization reaction was achieved by sandwiching GFPmut3* between the C-terminal 36-residue segment and N-terminal 123-residue segment of the *Ssp* DnaE intein. In 2001, Iwai and his co-workers [9] developed an artificially split intein system for production of circular protein *in vivo*. Unlike the majority of the artificially split inteins, *Ssp* DnaE intein fragments can mediate efficient protein splicing after reconstitution *in vitro* under native conditions [4]. *In vitro* modulation has been made possible by the addition of a chitin-binding domain (CBD) tag to the intein sequence [26]. This design permits the cyclization of GFPmut3* to proceed during expression of the fusion gene in *E. coli* host cells or following purification of the precursor protein on the chitin column *in vitro* (Fig. 1).

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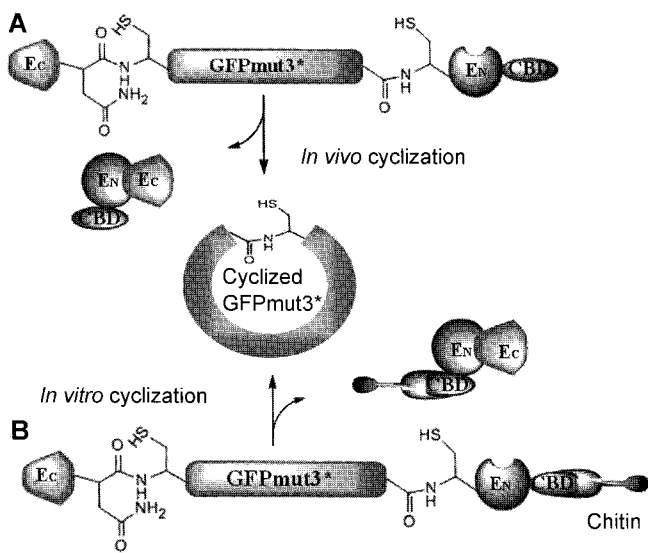


Fig. 1. Protein cyclization *in vivo* and *in vitro* using intramolecular *trans*-splicing activity of *Ssp* DnaE intein. GFPmut3* is sandwiched between the C-terminal 36-residue fragment (EC) and N-terminal 36-residue fragment (EN) of *Ssp* DnaE intein. Splicing mediates the ligation of the N and C termini of GFPmut3* through a native peptide bond. **A.** A circular GFPmut3* is formed by a *trans*-splicing reaction during protein expression in living cells. **B.** A chitin-binding domain fused to the C terminus of EN permits the purification of the precursor protein for subsequent cyclization of protein *in vitro*.

MATERIALS AND METHODS

Plasmid Construction

The DNA sequence encoding the C-terminal 36-residue segment (EC) followed by 5 native extein residues was amplified from pKEB1 (NEB) with primers 5'-AAGCTTGCCATGGTTAAAGTTATCGGTT-3' and 5'-CTTAAGACCGGTGGATATGTTAAAGCAGTTAGCTGCGATAGCGC-3', followed by digestion with *Hind*III and *Afl*II. The *gfpmut3** gene was amplified from the plasmid pTG [27] by polymerase chain reaction (PCR) with the primers 5'-CTTAAGCACATCATCATCATCATATGCGTAAAGGAGAAGAAC-3' and 5'-ATCGATGCTACCACGCGGAACCAAGTTGTATAGTTCATCCATG-3', followed by digestion with *Afl*II and *Clal*. Following a his-tag at the N terminus, a thrombin cleavage site was added to the C terminus of the *gfpmut3** gene. The N-terminal 123-residue segment of the *Ssp* DnaE intein with 5 native extein residues at its N terminus was amplified by annealing oligonucleotides 5'-ATCGATAACCTCGGGATCGAGGGAAGGGGACGCTCGAGAAATTTGC3' and 5'-GAATTCAGGGGCTCTTCCTTAATTGTCAGCGTCAA-G-3' from plasmid pMEB21 [4], followed by digestion with *Clal* and *Eco*RI. The three PCR-amplified fragments were ligated together and cloned into the *Hind*III/*Eco*RI sites in pMEB21, which contains the *Bacillus circulans* chitin-binding domain (CBD). The resulting plasmid, pGEB6th, expressed the EC-GFPmut3*-EN-CBD fusion protein under the control of the T7/lac promoter.

Protein Cyclization and Purification

Vector pGEB6th was transformed into *E. coli* ER2566. The cells were grown in 1 l of LB medium containing 100 μ g/ml ampicillin at 37°C to an $A_{600\text{nm}}$ of 0.5. Protein expression was induced by the

addition of IPTG to 0.3 mM final concentration at 15°C for 16 h. Cells were then harvested by centrifugation at 5,000 \times g for 10 min and lysed by sonication in buffer A (20 mM Tris-HCl, pH 7.0, with 500 mM NaCl and 1 mM EDTA). After centrifugation at 20,000 \times g for 30 min, the clarified supernatant was applied to a 2-ml chitin resin (NEB). Unbound proteins were washed from the column with 30 ml of buffer A. The intramolecular *trans*-splicing reaction proceeded *in vitro* when the column was incubated at 22°C for 17 h. Following incubation, the reaction products were eluted with buffer A. The fraction that was unbound to the chitin resin was applied to a 2-ml Ni-nitrilotriacetic acid (NTA) column (Qiagen Inc.) pre-equilibrated with buffer B (20 mM Tris-HCl, pH 7.9, with 500 mM NaCl and 10% glycerol). The column was washed with 10 ml of buffer C (20 mM Tris-HCl, pH 7.9, with 500 mM NaCl, 10% glycerol, and 20 mM imidazole). The products were eluted with buffer D (20 mM Tris-HCl, pH 7.9, with 500 mM NaCl, 10% glycerol, and 80 mM imidazole). Then, 100- μ l samples from each step were saved and analyzed by 12% SDS-PAGE. The cyclic GFPmut3* was analyzed by treatment with thrombin (1:100, thrombin:protein mass-to-mass ratio) at 23°C for 12 h to generate linearized cGFPmut3*. The concentration of the eluted protein was determined spectrophotometrically at 280 nm. Protein purity was estimated by comparing the intensity of samples run on 12% SDS-PAGE gel. The stained SDS-PAGE gel was quantified by gel document scanning with the Bio-Rad Quantity One 4.6.1 1-D Analysis software.

Final proteins treated with thrombin overnight were resolved by 12% SDS-PAGE and then transblotted onto polyvinylidene fluoride (PVDF) membrane (Millipore Corporation, Bedford, MA, U.S.A.), and the individual bands were subjected to amino acid sequencing.

Thermostability and Exopeptidase-Resistance Test

The purified protein solution in the reaction buffer A (20 mM Tris-HCl, pH 7.0, with 500 mM NaCl and 1 mM EDTA) was incubated at various temperatures (from 70 to 82°C). The insoluble fraction was removed by centrifugation at 20,000 \times g for 30 min. The supernatants were dissolved with sodium dodecyl sulfate (SDS) loading buffer. Each soluble fraction was analyzed by SDS-PAGE on a 12% Tris-glycine gel.

Carboxypeptidase Y (Worthington Biochemical Co., Lakewood, NJ, U.S.A.) was used to test the exopeptidase-resistance of circular and linear GFPmut3*. The purified proteins were digested by carboxypeptidase Y (1:10, carboxypeptidase Y:protein mass-to-mass ratio) at 25°C, pH 6.5, for 12 h. All samples were analyzed by electrophoresis on a 10% SDS-PAGE gel.

Confocal Microscopy Experiments

Transformed *E. coli* ER2566 cells expressing the circular GFPmut3* protein were washed three times with buffer A, and fluorescent images were collected using a Leica TCS SP2 laser scanning spectral confocal microscope (Germany) with an objective 100 \times oil immersion lens. The excitation wavelength was 488 nm, and the detection bandwidth was 500 to 600 nm.

Fluorescence Measurements

All fluorescence measurements reported in this study were done in an F-4500 Hitachi fluorescence spectrophotometer (Japan). A cuvette with 1-cm path length was used. The samples for the excitation and emission scans were prepared in 50 mM Tris-HCl, pH 7.8.

Unfolding Kinetic Measurements

The unfolding kinetic measurements were performed in solution containing 50 mM Tris-HCl, pH 7.8, and different GdnHCl concentrations. The final protein concentrations were 20 $\mu\text{g/ml}$ at 25°C. The processes were monitored by the change of the fluorescence intensity versus time.

Western Blot Analysis

Purified proteins were run on a 10% SDS-PAGE gel and transferred electrophoretically onto a PVDF membrane and probed with anti-his antibodies raised in mice, followed by chemiluminescent detection (Tiangen Biotech Co., Ltd., China).

RESULTS

Construction of Expression Vector

The *gfpmut3** gene was sandwiched between the C- and N-terminal fragments (EC and EN) of *Ssp* DnaE intein with five native *Ssp* DnaE C-extein residues at its N terminus and five *Ssp* DnaE N-extein residues at its C terminus to enhance the efficiency of the cyclization reaction. Furthermore, the PCR primers used to amplify *gfpmut3** introduced a six-histidine tag at the 5' end of *gfpmut3** to allow purification of circular GFPmut3* produced *in vivo*, and a thrombin cleavage site was added to the 3' end of *gfpmut3**. A chitin-binding domain affinity tag was fused to the C terminus of the EN sequence.

Production and Purification of Circular GFPmut3*

The resulting fusion gene in the construct, pGEB6th, was under the control of a T7/lac promoter. Expression of the EC-GFPmut3*-EN-CBD fusion protein was induced at 15°C for 16 h in the presence of IPTG. The cells induced at 15°C showed bright green fluorescent color under the confocal microscope (Fig. 2A). It was previously observed that production of active GFPmut3* requires low temperature for its expression [27]. When the strains were incubated at 4°C for several days without addition of IPTG, the

expression of GFPmut3* was still detectable (Fig. 2B). However, if the strains were induced by IPTG at 37°C for 2 h, no fluorescence indicative of a mature GFPmut3 chromophore was detected. SDS-PAGE analysis showed that a precursor protein was produced as an inclusion body (data not shown).

Following IPTG (0.3 mM)-induced protein expression at 15°C for 16 h, the cells were harvested by centrifugation and lysed by sonication. After removing the debris, the cell lysate was applied to a chitin resin for *in vitro* cyclization, and the *trans*-splicing reaction proceeded at 22°C, pH 7.0, for 17 h. Following incubation, the reaction products were eluted with buffer B (20 mM Tris-HCl, pH 7.0, with 500 mM NaCl and 1 mM EDTA). Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis showed that there were two major bands with molecular mass of about 30 kDa (Fig. 3A, lane 2). Western blot assay indicated that the two bands were GFPmut3* species (data not shown). Both bands were electroblotted onto a PVDF membrane and confirmed by amino acid sequencing. The N-terminal sequence of the upper band was CFNISTGLKCLKHHH, which is the N terminus of the precursor form of linear GFPmut3*. The N-terminal sequence of the lower band was not detected, and thus we presumed that this species was circular GFPmut3*, probably because of cross-linking of the N and C termini. After treatment with thrombin, two species were detected (Fig. 3A, lane 3). The N-terminal sequence of the upper band (linearized cGFP) that migrated slowly was GSIDNLGIEGRGTLEKFA, suggesting that it was generated by linearizing the circular GFPmut3* by thrombin. The linear form of GFPmut3* (l-GFP) released an amino acid peptide from its C terminus after thrombin digestion (Fig. 3B). The truncated l-GFP, which has the same N-terminal sequence as linear GFPmut3*, showed an apparent increase in electrophoretic mobility (Fig. 3A, lane 3). The linearized cGFPmut3* displayed a decrease electrophoretic mobility. The yield of proteins was 0.54 mg from 1 l of bacterial culture. The two species of GFPmut3*

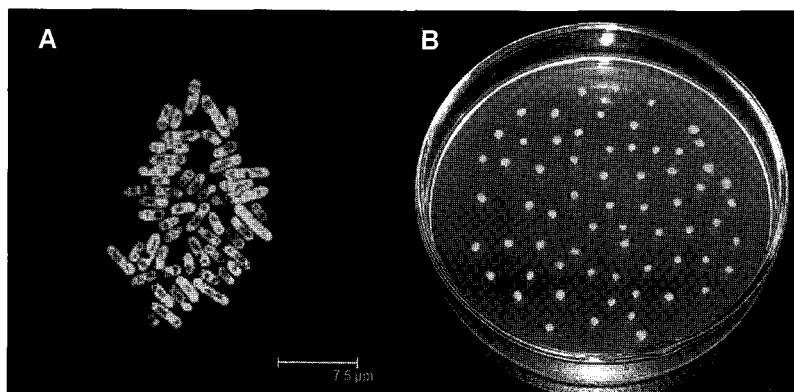


Fig. 2. A. Fluorescent imaging of *E. coli* T7 expressed cells transformed by pGFP6th; B. Visible green colony of *E. coli* with GFPmut3* fusion protein expression.

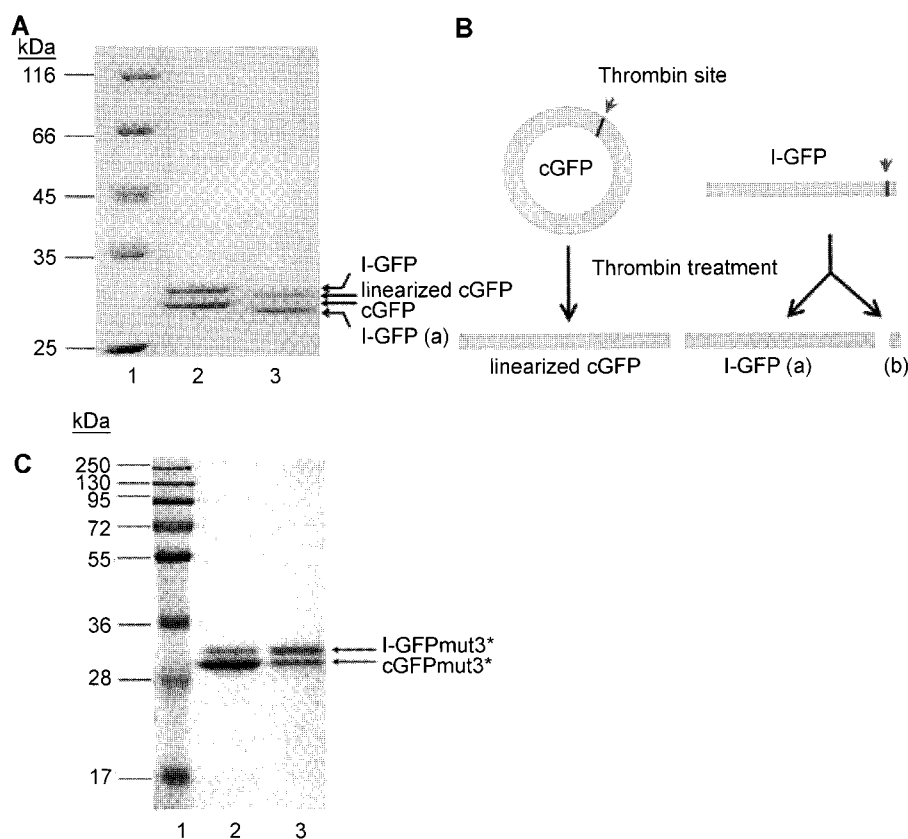


Fig. 3. A. Examination of purified GFPmut3* on chitin column by SDS-PAGE.

Lane 1, protein marker; Lane 2, proteins eluted from chitin resin following incubation at 22°C for 17 h; Lane 3, incubation of eluted proteins with thrombin (1:100, thrombin:GFPmut3*). **B.** Diagram illustrating the proteolytic treatment on circular GFPmut3* (cGFP) and linear GFPmut3* (I-GFP) by thrombin. **C.** Protein cyclization *in vivo* and *in vitro*. Lane 1, protein marker; Lane 2, proteins eluted from NTA resin; Lane 3, proteins eluted from chitin resin *in vitro* cyclization.

were quantified by Bio-Rad quantity analysis software on SDS-PAGE gel. The assays revealed that the ratio of cGFPmut3* to I-GFPmut3* was 1.38.

The flow-through fraction unbound to the chitin resin was loaded onto a Ni-nitrilotriacetic acid (Ni-NTA) column for purification of the cyclized GFPmut3* generated *in vivo*. The majority of the GFPmut3* fusion protein was processed during expression, yielding mostly the circular GFPmut3* product, and by-product of the linear species still existed (Fig. 3C, lane 2). The yield was 4.74 mg from 1 l of bacterial culture. The ratio of cGFPmut3* to I-GFPmut3* *in vivo* was 3.35.

Stability of the Cyclized Proteins

Cyclization of a protein could, in principle, increase the stability and resistance to exopeptidase. Carboxypeptidase Y has broad amino acid specificity, which makes it a suitable exopeptidase for testing protease resistance. The mixture of purified proteins was treated with carboxypeptidase Y at 25°C, pH 6.5, for 12 h. SDS-PAGE analysis demonstrated that the linear form of GFPmut3* (I-GFP) was degraded by proteolytic cleavage, yielding a degraded linear GFPmut3*

(d-GFP) with more rapid migration by electrophoresis (Fig. 4A). The circular form of GFPmut3* (cGFP) exhibited no degradation under the same proteolytic treatment with carboxypeptidase Y, suggesting that the termini of this GFPmut3* species were joined *via* a peptide bond, conferring resistance to exopeptidase treatment.

To test resistance against heat precipitation, the mixture of circular and linear forms of GFPmut3* was incubated at various temperatures from 70°C to 82°C for 10 min. After the removal of the precipitate by centrifugation, the soluble fractions were analyzed on 12% SDS-PAGE gel by quantity analysis software. At 78°C, essentially all the linear form of GFPmut3* precipitated, but only 50% of the circular form of GFPmut3* precipitated under the same condition (Fig. 4B). The circular form of GFPmut3* entirely denatured at 82°C, and thus showed a 5°C increasing stability against heat precipitation compared with the linear form. The data indicate that cyclization enhanced the thermostability of GFPmut3*.

The unfolding kinetic properties of the circular and linearized GFPmut3* were measured as the fluorescence intensity versus time in 7.0 M GdnHCl (Fig. 5B). The

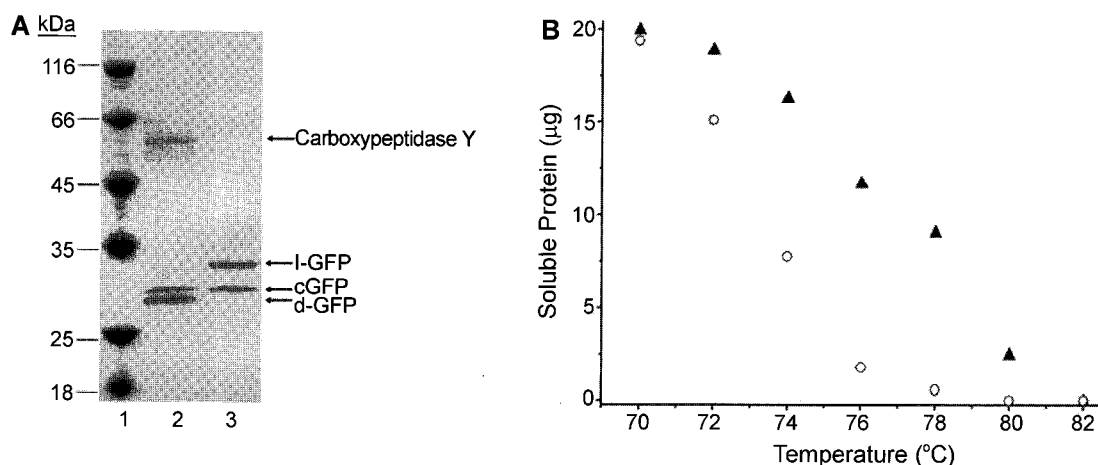


Fig. 4. A. Proteolysis resistance of circular GFPmut3*.

Lane 1, protein marker; Lane 2, incubation of the eluted proteins with carboxypeptidase Y at 25°C for 12 h; Lane 3, eluted proteins before addition of carboxypeptidase Y. **B.** Effect of heat treatment at different temperatures on circular GFPmut3* (▲) and linear GFPmut3* (○) solubility.

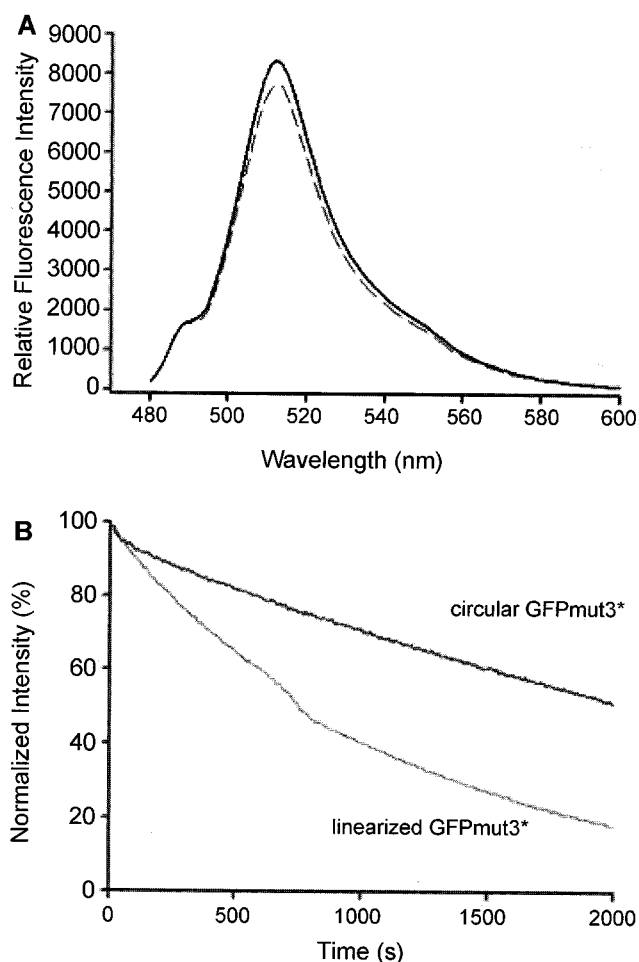


Fig. 5. A. Emission spectra of circular GFPmut3* (solid line) and linearized GFPmut3* (dashed line).

The emission maxima was 512 nm. **B.** Unfolding kinetics of the circular GFPmut3* (black line) and linearized GFPmut3* (grey line). The measurements were performed at 25°C and pH 7.8, with a final concentration of 7.0 M GdnHCl.

excitation and emission wavelengths were 501 nm and 510 nm, respectively. The spectroscopic characterization of GFPmut3* is a result of both its chromophore formation and folded structure. The data showed that the circular GFPmut3* unfolded more slowly than the linearized form, which suggests that cyclization makes the protein more difficult to unfold and a circular protein would be more stable against chemical denaturation.

Spectroscopic Characterization of Circular GFPmut3*

After removing the linear GFPmut3* by heat precipitation, the circular GFPmut3* was treated with thrombin. Then, the fluorescence emission spectra of circular GFPmut3* and linearized cGFPmut3* were recorded by fluorescence spectrophotometry. Both types of GFPmut3* showed an emission maxima of 512 nm, close to the reported 511 nm [2]. However, we found that the circular GFPmut3* showed increased maximum values of relative fluorescence intensity compared with the linearized form (Fig. 5A), which were 8,378 and 7,744, respectively. Circular GFPmut3* and the linearized form have the same peptide linker, and cyclization would not change the structure and the core conformation of GFP [7]. We presume that the enhancement in fluorescence intensity may be due to the reduced unfolding rate of the circular protein (Fig. 5B). The unfolding process of circular GFPmut3* was slower than the linear form. The result implies that cyclization may confer conformational constraint, which may contribute to higher biological activity as well as higher stability [8].

DISCUSSION

Some protein cyclization approaches *in vitro* require generation of a C-terminal thioester intermediate by chemical

treatment [3, 10]. In the present study, the cyclization reaction was mediated efficiently *in vitro* under native conditions without costly chemical and multistep manipulation. After loading the cell lysate onto the chitin column, the fusion precursor protein bound onto the column, which allowed the cyclization reaction to proceed *in vitro*. Compared with the majority of the artificially split inteins, this system can mediate controllable cyclization *in vitro*. Furthermore, the inclusion of two small protein tags, a chitin-binding domain and a six-histidine tag, aids in characterization of *in vivo* and *in vitro* cyclization reactions. The *trans*-splicing reaction proceeded with the precursor protein bound onto chitin beads *in vitro*. *Ssp* DnaE intein also mediated efficient protein cyclization *in vivo*, where cyclic protein was produced in living cells. Optimal expression of GFPmut3* was carried out at 15°C for 16 h. Most of the precursor proteins were already processed *in vivo* at this temperature, because splicing appeared more efficient at 15–25°C, as reported earlier [26]. However, expression of another fusion protein induced at a less permissive temperature (*e.g.*, 37°C) may slow down processing of the protein precursors and increase the yield of the final cyclic protein product in the *in vitro* cyclization experiment. Future studies may focus on improving the efficiency of cyclization and decreasing the formation of linear by-products.

The intein N- and C- terminal segments (EN and EC) mediated the ligation of the N and C termini of GFPmut3* by a native peptide bond. Proteins whose termini are in reasonably close proximity in their native structures are amenable for cyclization. The N and C termini of proteins are often close together and surface exposed [21, 22]. If the structure of the target protein was unknown, a longer peptide linker may be used for cyclization. The distance of the N and C termini of GFPmut3* was 2.86 nm (PDB code 1qyo). A 39-residue peptide linker was introduced between the two termini. The results indicate that this peptide linker had no negative impact on GFPmut3*. The circular GFPmut3* had the same spectroscopic characteristics as the reported GFPmut3*, and its fluorescence intensity was significantly enhanced. This indicates that the circular protein can tolerate the insertion of a long peptide linker with no change in the characteristics of the protein. In general, the choice of the peptide linker, including the quantity and types of amino acid residues, needs serious consideration. The peptide linker should provide the flexibility to ensure the activity of the cyclized product [24].

Increased resistance to exopeptidase digestion and improved thermostability are two benefits for applications in protein engineering. Circular proteins lacking free N and C termini are less susceptible to attack by exopeptidase. Cyclization could add the conformational constraints, dramatically slowing down the unfolding rate of the protein [5, 6, 17], which contributes to enhanced stability.

These benefits make backbone cyclization an attractive tool for improving the *in vivo* and *in vitro* stability of protein of interest, in particular, therapeutic proteins and industrial enzymes.

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