

Research Article

A Novel Method for High-Level Production of TEV Protease by Superfolder GFP Tag

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Because of its stringent sequence specificity, tobacco etch virus (TEV) protease is widely used to remove fusion tags from recombinant proteins. Due to the poor solubility of TEV protease, many strategies have been employed to increase the expression level of this enzyme. In our work, we introduced a novel method to produce TEV protease by using visible superfolder green fluorescent protein (*sfGFP*) as the fusion tag. The soluble production and catalytic activity of six variants of *sfGFP*-TEV was examined, and then the best variant was selected for large-scale production. After purified by Ni-NTA affinity chromatography and Q anion exchange chromatography, the best variant of *sfGFP*-TEV fusion protease was obtained with purity of over 98% and yield of over 320 mg per liter culture. The *sfGFP*-TEV had a similar catalytic activity to that of the original TEV protease. Our research showed a novel method of large-scale production of visible and functional TEV protease for structural genomics research and other applications.

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1. Introduction

Nowadays, it has been a popular way to fuse target proteins with various tags to facilitate expression and purification. An efficient combination of solubility-enhancing tags, such as maltose-binding protein (MBP) [1, 2], N-Utilization substance (NusA) [3], glutathione S-transferase (GST) [4], thioredoxin (TRX) [5], trigger factor [6], and SUMO [7], will promise high-throughput expression and purification methods for many target proteins and sometimes increases their solubility. However, these fusion tags may become a drawback for further structural and functional studies [8]. Therefore, the removal of these tags is necessary in many situations. Proteases such as enterokinase, thrombin, and factor Xa [9] as well as the more specific human rhinovirus 3C protease (3CP or PreScission [10]) and tobacco etch virus (TEV) protease [11] can fulfill the task to liberate fusion tags from target proteins.

The widely used TEV protease is the 27 kDa catalytic domain of the nuclear inclusion an (NIa) protease from tobacco etch virus [12]. Among various proteases, TEV protease outstands because of its high and unique specificity. It can recognize the canonical cleavage site, ENLYFQ/G

[11] and the P1' position can tolerate substitutions with small amino acids [13]. Moreover, TEV protease can be used at temperature as low as 4°C with adequate efficiency to reduce the proteolysis of the target protein. Because of these advantages, nowadays, it is used more frequently than other proteases (enterokinase, thrombin, factor Xa, and human rhinovirus 3C protease) in structural genomic research projects.

Production of TEV protease in *E. coli* has been problematic due to its low solubility. To increase its soluble production, many strategies have been addressed. First, Kapust et al. [14] designed a more stable mutant of TEV protease named S219V. van den Berg et al. [15] obtained a mutant TEV_{SH} with production of 54 mg/L culture by directed evolution. Later, Fang et al. [16] increased the production to 65 mg/L culture using chaperone coexpression and low-temperature expression methods. More recently, Blommel and Fox [17] reported a combined approach raising the production to 400 mg/L culture while Kraft et al. developed a fluorogenic substrate which was useful to determine the TEV protease's expression and folding in vivo [18].

Fluorescent protein is widely used as gene reporter and protein marker, and so forth. However, existing variants of

green fluorescent protein (GFP) often misfold when fused to other proteins. Pédrelacq et al. [19] reported a robustly folded GFP called “superfolder GFP” (*sfGFP*) which could fold well regardless of the folding status or solubility of its fusion partner in *E. coli*. Furthermore, *sfGFP* fusions are more soluble than conventional GFP fusions.

In our present work, considering the high thermodynamic stability, robust folding kinetics, and solubility of *sfGFP* fusions, we tempted to fuse *sfGFP* to TEV protease hoping that *sfGFP* would increase the soluble production of TEV protease. In order to minimize the possible steric hindrance of *sfGFP* that might decrease the activity of TEV protease, we further constructed 6 variants of *sfGFP*-TEV with different linkers of various lengths and composition between *sfGFP* and TEV. Then, the catalytic activity of *sfGFP*-TEV variants was tested and compared with that of the original TEV protease without *sfGFP* tag. Finally, we obtained one variant of *sfGFP*-TEV fusion protease with soluble production of over 320 mg/L culture. Compared with the original TEV protease, this variant of *sfGFP*-TEV has similar catalytic activity and is easy for detection during expression, purification, and applications because of the presence of green fluorescence. The results of our work also present the potential of superfolder GFP to become a solubility-enhancing fusion tag with fluorescence.

2. Materials and Methods

2.1. Materials. The bacterial hosts, *E. coli* DH5 α , Rosetta (DE3) pLysS, and the vector pET21a were obtained from Novagen (Madison, WI). KOD Plus polymerase and the DNA ligation kit were purchased from Toyobo (Osaka, Japan). Nucleotides, agarose gel, the DNA extraction kit, and the PCR purification kit were purchased from Roche Diagnostics (Indianapolis, IN). Primer synthesis and DNA sequence analysis were performed by Invitrogen (Shanghai, China). Restriction endonucleases were purchased from Takara (Dalian, China). The nickel-nitrilotriacetic acid (Ni-NTA) superflow matrix was obtained from Qiagen (Chatsworth, CA). Q Sepharose Fast Flow was from GE Health (Sweden). Amylose Sepharose was purchased from New England Biolabs (Hitchin, UK). Bicinchoninic acid (BCA) Protein Assay Reagent Kit was from Pierce (Rockford, IL). Imidazole, D-glucose, and D-lactose were from Sigma (St Louis, MO). All other agents are of analytic purity. PRK793-TEV expression vector was a gift from Dr. Waugh [14].

2.2. Construction of *sfGFP*-TEV and TEV Expression Vectors. We have previously reconstructed an expression vector, designated pT7His, which contained the N-terminal His₁₀ and C-terminal His₆ tags from the vector pET21a. The detailed vector construction procedure was similar to that of pT7470 with N-terminal His₆ and C-terminal His₆ tags [20]. We optimized the codon usage of superfolder GFP's cDNA by referring to its amino acid sequence [19]. The whole gene synthesis of superfolder GFP was accomplished by 2 rounds PCR with 18 central primers listed in Table 1, one

5' primer 5'-GATATACATATGAGCAAAGGCCGAAGAA-3' and one 3' primer 5'-GCCGGATCCGCCCCCGGAACCCCTCCGTTATTGTTATTCTTGTACAGCTCGTCCAT-3'. Considering that the C-terminal poly (R) in PRK793-TEV would decrease the solubility of TEV protease [17], we replaced the poly (R) with residue E to construct the plasmid TEV 238 Δ by PCR with primers 5'-GGGGGTAGCGGC-GGTGGCAGCGGCGGAGAAAGCTTGTTTAAAG-3' and 5'-TTACTCGAGTCATTCATTCATGAGTTGAGTCGC-3'. We have constructed 6 recombinant *sfGFP*-TEV fusion proteins with different linkers. The linker region of *sfGFP*-TEV-His₆ Nd1-6 was listed in Table 2. The plasmid TEV 238 Δ was also used as the PCR template to produce the control TEV protease. The PCR product was incorporated into the expression vector MBP-LTL-His₆ [21]. The final expression vector MBP-LTL-TEV-His₆ which produced TEV-His₆ (MBP tag was self-cleaved during expression) was employed as a control in further experiments.

2.3. Expression of *sfGFP*-TEV, TEV-His₆, and MBP-EGFP. The expression vectors mentioned above were transformed into *E. coli* strain Rosetta (DE3) pLysS. After the colony had grown overnight at 37°C in 5 mL of LB medium with 100 μ g/mL ampicillin, 0.5 mL of the bacterial suspension was transferred into a 2L flask containing 250 mL autoinduction medium. (For 1 liter culture, we used 4 flasks to ensure the sufficient oxygen supply). The autoinduction medium was prepared as studier's original protocol [22]. Standard stock solutions include 20*P (1 M Na₂HPO₄, 1 M KH₂PO₄, and 0.5 M (NH₄)₂SO₄), 50*M (1.25 M Na₂HPO₄, 1.25 M KH₂PO₄, 2.5 M NH₄Cl, and 0.25 M Na₂SO₄), and 50*5052 (25% glycerol, 2.5% glucose, and 10% D-lactose); the working autoinduction medium was assembled by adding sterile concentrated stock solutions into sterile water. When the cells had grown (250 rpm) at 37°C to an optical density at 600 nm (OD₆₀₀) of 0.6 (around 3 hours), the cells were cooled to 19°C and shaken at 250 rpm for 20 hours. Finally, the cells were collected by centrifugation at 6,000 \times g for 20 minutes and stored at -80°C. In order to reflect the real-time expression level of *sfGFP*-TEV, the induced *E. coli* cells in the autoinduction medium were collected at 0, 2, 4, 6, 8, 10, 12, 14, and 16 hours, respectively. The fluorescence of 100 μ L *E. coli* cells in the 96-well plates was recorded by DTX 880 multimode detector (Beckman) using bottom reading method with 485 nm excitation filter and 535 nm emission filter.

2.4. Purification of *sfGFP*-TEV and TEV-His₆. The *sfGFP*-TEV-His₆ Nd1-6 recombinant proteins were all first purified by Ni-NTA affinity chromatography. The frozen cell pellet was thawed and resuspended in Buffer A (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 10% [v/v] glycerol, 20 mM imidazole). Then, the cells were lysed by sonication on ice and the lysate was cleared by two-round 20-minute centrifugation at 20,000 \times g. The retained supernatant was loaded onto a Ni-NTA Superflow column which was pre-equilibrated with Buffer A. After loading, the Ni-NTA column was washed

TABLE 1: Primers for whole gene synthesis of superfolder GFP.

No. and length	Oligo Sequences (5' → 3')
S1F 59	ATGAGCAAAGGCGAAGAAGCTGTTTACCGCGTGGTGCCGATTCTGGTTGAACTGGATGG
S2R 59	GCCTTCGCGCGCACGCTAAACTTATGGCCATTAACATCGCCATCCAGTTCAACCAGAA
S3F 59	TAGCGTGC GCGGCGAAGGCGAAGGCGATGCGACCAACGGCAAACCTGACCCTGAAGTTTA
S4R 59	AGGGTCCGCCACGGCACCGGAGTTTGCCGGTGGTGCAATAAACTTCAGGGTCAGTTT
S5F 59	CGGTGCCGTGGCCGACCCTGGTGACCACCTGACCTATGGCGTGCAAGTCTTTAGCCGC
S6R 59	CGGACTTAAAGAAATCATGGCGTTTCATGTGATCCGGATAGCGGCTAAAGCACTGCACG
S7F 59	CATGATTTCTTTAAGTCCGCGATGCGGAAAGGCTATGTTCAAGAACGCACCATTAGCTT
S8R 59	TTTCACTTCGCGCGGGTCTTATAGGTGCCATCATCTTTAAAGCTAATGGTGGCTTCCT
S9F 59	GACCCGCGCGAAGTAAAATTTGAAGGCGATACCTGGTGAACCGCATTGAACTGAAAG
S10R 59	TTATGGCCAGAATGTGCCATCTTCTTTGAAGTCAATGCCTTTCAGTTCAATGCGGTT
S11F 59	GCAACATTCTGGCCATAAACTGGAGTACAATTTCAACAGCCATAACGTGTATATTACC
S12R 59	TTTTGAAATTCGCTTTGATGCCGTTCTTCTGTTTGCCGGTAATATACACGTTATGG
S13F 59	ATCAAAGCGAATTTCAAATCCGCCATAATGTGGAAGATGGCAGCGTGCAGCTGGCGGA
S14R 59	CACCGGGCCATCGCGATCGGGGTATTCTGCTGATAGTATCCGCCAGCTGCACGCTGC
S15F 59	GATCGGCGATGGCCCGGTGCTGCTGCCGACAATCACTACCTAGCAGCCAGTCCGTCG
S16R 54	CATGTGATCGCGTTTCTCATTCCGGATCTTTGCTCAGCAGCGACTGGGTGCTCAG
S17F 54	TGAGAAACGCGATCACATGGTTCTGCTGGAGTTTGTGACCGCGCGGGTATCAC
S18R 44	CTGTACAGCTCGTCCATGCCATGAGTGATACCCGCCGCGTCA

with Buffer A with 40 mM imidazole. The column was equilibrated again with Buffer A and then eluted with Buffer B (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 10% [v/v] glycerol, 500 mM imidazole). The fluorescence of 100 μ L Ni-NTA purification sample was also recorded by DTX 880 multimode detector (Beckman) using top reading method with 485 nm excitation filter and 535 nm emission filter.

The collected elution from Ni-NTA affinity chromatography was immediately diluted with 5 volume QA (50 mM Tris-HCl [pH 8.0], 10% [v/v] glycerol). The dilution was loaded onto a Q Sepharose Fast Flow column pre-equilibrated with QA. After washed with QA, the protein was eluted with a linear 0–0.9 M NaCl gradient by automatically mixing QA and QB (50 mM Tris-HCl [pH 8.0], 1 M NaCl, 10% [v/v] glycerol). Fractions were analyzed by SDS-PAGE and quantified by BandScan 4.30 (Glyko) and were pooled based on their purity and concentration. The concentration of pooled protein sample from Q anion exchange chromatography and elution sample from Ni-NTA affinity chromatography was determined by BCA method according to the reagent kit protocol (Pierce). The pooled protein was dialyzed in dialysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.5 mM EDTA, 10% [v/v] glycerol) at 4°C and then diluted with storage buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.5 mM EDTA, 80% [v/v] glycerol) to a protein concentration of ~2 mg/mL in 40% glycerol. The purified protein was finally stored at –20°C.

TEV-His₆ was purified by Ni-NTA affinity chromatography using the similar methods described above. The purified protein was dialyzed in dialysis buffer and diluted with storage buffer to a protein concentration of ~1 mg/mL in 40% glycerol. The purified TEV-His₆ was stored at –20°C.

2.5. Purification of TEV Protease Substrate MBP-EGFP. For the purification of MBP-EGFP, the cell pellet was thawed and resuspended in Amylose A buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 10% [v/v] glycerol). After sonication, the supernatant was retained by two-round 20-minute centrifugation at 20,000 \times g and then loaded onto the Amylose Sepharose Column pre-equilibrated with Amylose A buffer. MBP-EGFP was eluted with Amylose B buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 10% [v/v] glycerol, 20 mM maltose). The purified protein sample was analyzed by SDS-PAGE and quantified by BandScan 4.30 (Glyko). The purified MBP-EGFP was dialyzed in dialysis buffer (150 mM NaCl, 10% [v/v] glycerol, 1 mM EDTA) at 4°C and then stored at 4°C.

2.6. Activity Assay of sfGFP-TEV and TEV-His₆. The catalytic activity of sfGFP-TEV-His₆ Nd1–6 and TEV-His₆ was determined by cleaving the substrate MBP-EGFP which contained a TEV cleavage site between MBP and EGFP. Prior to activity assay, the protein concentration of sfGFP-TEV-His₆ Nd1–6, TEV-His₆, and MBP-EGFP was determined by BCA method according to the reagent kit protocol (Pierce). The time course assay was conducted at 17°C for a given incubation time (0, 5, 10, 20, 40, 60, 90, 120, 180, and 240 minutes, respectively). The mass ratio of substrate to enzyme (calculated by the mass of effective TEV protease) is 100 : 1. At any given time, the reaction was stopped by adding 3 \times loading buffer (150 mM Tris-HCl [pH 6.8], 300 mM DTT, 6% [w/v] SDS, 0.06% [w/v] bromophenol blue, 30% [v/v] glycerol). The samples were boiled at 95°C for 3 minutes and then loaded onto 12% SDS-PAGE gel for electrophoresis. After visualized by staining with Coomassie G-250, the gel was quantified by BandScan 4.30 (Glyko) to establish the

TABLE 2: Linker region of *sfGFP-TEV-His₆* Nd1–6.

Construction Code	Abbreviation	Linker length (aa)	Anticipated linker composition
<i>SfGFP-TEV-His₆</i> Nd1	Nd1	2	...THG/GS/RD...
<i>SfGFP-TEV-His₆</i> Nd2	Nd2	5	...THG/GSKGP/RD...
<i>SfGFP-TEV-His₆</i> Nd3	Nd3	8	...THG/NNPGSKGP/RD...
<i>SfGFP-TEV-His₆</i> Nd6	Nd6	8	...THG/GSNLFGKP/RD...
<i>SfGFP-TEV-His₆</i> Nd4	Nd4	11	...THG/NNPGSNLFGKP/RD...
<i>SfGFP-TEV-His₆</i> Nd5	Nd5	14	...THG/MDPNPGSNLFGKP/RD...

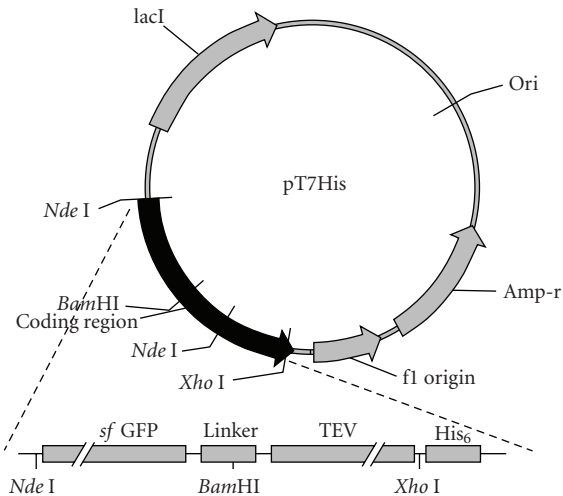


FIGURE 1: Maps of expression vector for *sfGFP-TEV-His₆* Nd1–6 used in our work. The vector map was created by Vector NTI software. All variants of *sfGFP-TEV-His₆* use the same vector pT7His derived from pET21a. The coding region in black will produce *sfGFP-TEV-His₆* Nd1–6.

time-course curve. The reaction condition was 75 mM NaCl, 0.5 mM EDTA, 25 mM Tris-HCl 8.0, and 10% [v/v] glycerol.

3. Results and Discussion

3.1. Construction of Expression Vector for *sfGFP-TEV* and *TEV*.

In order to maximize the expression level of the recombinant *sfGFP-TEV* proteases, we first synthesized the *sfGFP* gene according to the synonymous codon choice which is optimal for the *Escherichia coli* translational system. Figure 1 shows the vector map we used for high-level expression of *sfGFP-TEV-His₆* Nd1–6. The *sfGFP-TEV* coding sequence was cloned to the pET derived vector pT7His which possesses the strong bacteriophage T7 promoter, ensuring the high level expression of target protein. Considering that the linker between *sfGFP* and *TEV* might have effects on the stability and catalytic activity of fusion protease, we constructed 6 variants of *sfGFP-TEV-His₆* with different linkers. The linker here is defined as the peptide between C-terminus of *sfGFP* “THG” and N-terminus of *TEV* “RDYNP.” The composition of different linkers with lengths varying from 2 to 14aa could be referred to Table 2. We also incorporated

a small peptide “GGG” at the C-terminus of *TEV*; so the C-terminuses of *sfGFP-TEV-His₆* Nd1–6 and *TEV-His₆* are all “LMNEGGGLEHHHHHH.” Our first attempt of *sfGFP-TEV* vector construction did not include the GGG small peptide between *TEV* and C-terminus *His₆* tag. However, during the Ni-NTA purification step, more than 70% expressed fusion protein did not bind with the Ni-NTA resin (data not shown). Perhaps the steric structure of *TEV* hindered *His₆* tag from binding with Ni-NTA resin. So we added the flexible GGG peptide between *TEV* and *His₆* tag. Almost all of the new version fusion protein can bind with Ni-NTA in the buffer containing relatively high concentration (20 mM) of imidazole.

3.2. Fusion of *sfGFP* to *TEV* Greatly Increases the Soluble Production of *TEV* Protease.

After autoinduction, *sfGFP-TEV-His₆* Nd1–6 were all purified by Ni-NTA affinity chromatography and Q anion exchange chromatography. After purification, there was an obvious main band around the molecular weight of 53 kDa (Figures 2(a) and 2(b)). Table 3 summarizes the purification results from 1-L culture medium. According to BandsScan software analysis, all variants of fusion protease were obtained with over 96% purity. Among them, Nd2, Nd4, and Nd5 were purified with over 98% purity. With the fusion of *sfGFP*, all variants could be purified by two-step chromatography with soluble production of over 200 mg. In particular, we could obtain around 320 mg of *sfGFP-TEV* Nd2 from 1-L culture medium. Because the molecular weight of *sfGFP-TEV* Nd2 and *TEV-His₆* was 53.8 kDa and 28.8 kDa, respectively, 320 mg/L of *sfGFP-TEV* Nd2’s effective *TEV* composition was close to 171 mg/L ($320 * 28.8/53.8 = 171$) of *TEV-His₆*. We also constructed the control expression vector for *TEV* protease without any tags, but there was almost no detectable *TEV* protease expressed under the same induction condition (data not shown). Therefore, the fusion of *sfGFP* to *TEV* significantly increases the soluble production of *TEV* protease.

3.3. Purification of *TEV-His₆* and *MBP-EGFP*.

We have also expressed and purified *TEV-His₆* as control and *MBP-EGFP* as *TEV* protease’s substrate. During expression, the *MBP* tag of *MBP-LTL-TEV-His₆* would be cleaved and then *TEV-His₆* was released. By Ni-NTA affinity chromatography and further dialysis, about 140 mg of *TEV-His₆* could be obtained from 1-L culture medium with around 98% purity. The

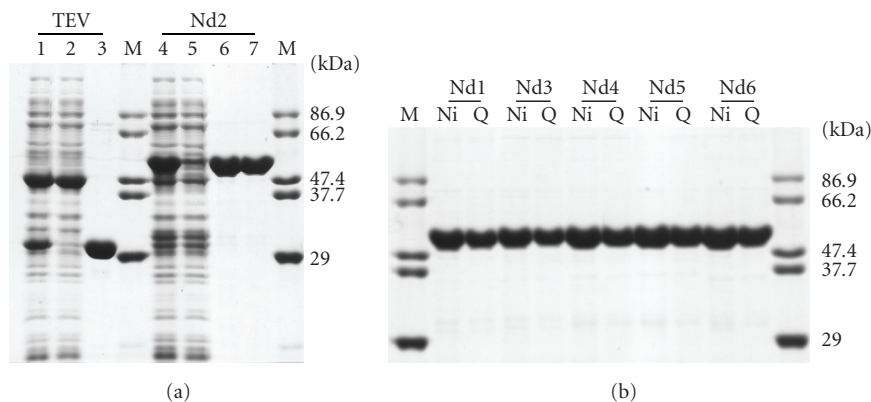


FIGURE 2: SDS-PAGE analysis of the expression and purification of *sfGFP-TEV-His₆* Nd1–6 and TEV-His₆. (a) Lanes 1–3 are the result for TEV-His₆: Lane 1: autoinduced whole-bacterial lysate; Lane 2: flow through from Ni-NTA affinity chromatography; Lane 3: the purified TEV-His₆ eluted from Ni-NTA affinity chromatography. Lanes 4–7 are the result for *sfGFP-TEV-His₆* Nd2: Lane 4: autoinduced whole-bacterial lysate; Lane 5: flow through from Ni-NTA affinity chromatography; Lane 6: the purified *sfGFP-TEV-His₆* Nd2 eluted from Ni-NTA affinity chromatography; Lane 7: the pooled sample of purified *sfGFP-TEV-His₆* Nd2 eluted from Q anion exchange chromatography. (b) The results for *sfGFP-TEV-His₆* Nd1 and Nd3–6. “Ni” represents the results of the purified protein eluted from Ni-NTA affinity chromatography; “Q” represents the pooled sample of purified protein eluted from Q anion exchange chromatography. “M” in (a) and (b) represents the protein marker.

TABLE 3: Purification results of *sfGFP-TEV-His₆* Nd1–6 and TEV-His₆ collected from 1-L of expression culture medium.

Abbreviation	Theoretical MW (kDa)	Total protein after Ni-NTA (mg)	Purity	Total protein after High Q (mg)	Purity	Total activity ($\mu\text{mol/h}$)	Specific activity ($\mu\text{mol/h/mg}$)
Nd1	53.5	244 \pm 1	90%	221 \pm 2	96%	7.3 \pm 0.2	0.067 \pm 0.002
Nd2	53.8	334 \pm 2	94%	323 \pm 1	98%	13.7 \pm 0.3	0.085 \pm 0.002
Nd3	54.1	220 \pm 4	93%	211 \pm 3	97%	8.0 \pm 0.1	0.076 \pm 0.001
Nd4	54.5	304 \pm 6	93%	293 \pm 4	98%	11.9 \pm 0.5	0.082 \pm 0.003
Nd5	55.0	299 \pm 1	93%	288 \pm 2	98%	11.3 \pm 0.6	0.079 \pm 0.005
Nd6	54.2	308 \pm 5	93%	300 \pm 4	96%	12.0 \pm 0.5	0.080 \pm 0.003
TEV	28.8	140 \pm 3	98%	—	—	12.6 \pm 0.6	0.090 \pm 0.005

electrophoresis results show that molecular weight of TEV-His₆ is around 29 kDa (Figure 2(a)). The substrate MBP-EGFP could also be purified with over 95% purity by Amylose affinity chromatography.

3.4. Cleavage Activity Assay of *sfGFP-TEV* and TEV. The cleavage activity assay of *sfGFP-TEV-His₆* Nd1–6 and TEV-His₆ could be determined by cleaving the substrate MBP-EGFP at the cleavage site “ENLYFQ/G” between MBP and EGFP. By SDS-PAGE, the remaining MBP-EGFP could be separated sufficiently with released MBP and EGFP (Figure 3(a)). After we set the quantity of MBP-EGFP at 0 min as 100%, the time course curve could be plotted by quantitatively analyzing the digested MBP-EGFP at the given time. Figure 3(b) shows the time course curve of *sfGFP-TEV-His₆* Nd1–6, TEV-His₆, and 2% TEV-His₆. Compared with the time course curve of TEV-His₆, we found that *sfGFP-TEV-His₆* Nd1–6 had different degrees of loss of catalytic activity. Among them, Nd2 had the closest curve to TEV-His₆. Ranking the cleavage rate at 60 minutes, the second highest ranked Nd2 could digest around 66% substrate,

which retained about 95% catalytic activity of TEV-His₆. Moreover, TEV-His₆ and all variants of *sfGFP-TEV-His₆* except Nd1 could efficiently cleave over 98% substrate after incubation for 4 hours at 17°C. However, the control 2% TEV-His₆ could only cleave less than 7% substrate under the same condition (Figure 3(c)). In conclusion, *sfGFP-TEV-His₆* Nd2 retained the most of catalytic activity among all variants.

Fusion tags are widely used to facilitate protein expression and purification. However, due to its drawback in structural and functional studies, these tags always need to be removed by various proteases. TEV protease is an ideal protease receiving most attention, thanks to its high specificity as well as toleration of a wide range of temperatures and presence of detergents [23]. One bottleneck for TEV protease is low soluble production due to its poor solubility. Researchers have tried many strategies including in silico design [24], direct-evolution [15], or coexpression with chaperone to increase its soluble production. These efforts have raised the production from 1 mg/L to 65 mg/L culture [16]. More recently, Blommel and Fox reported a

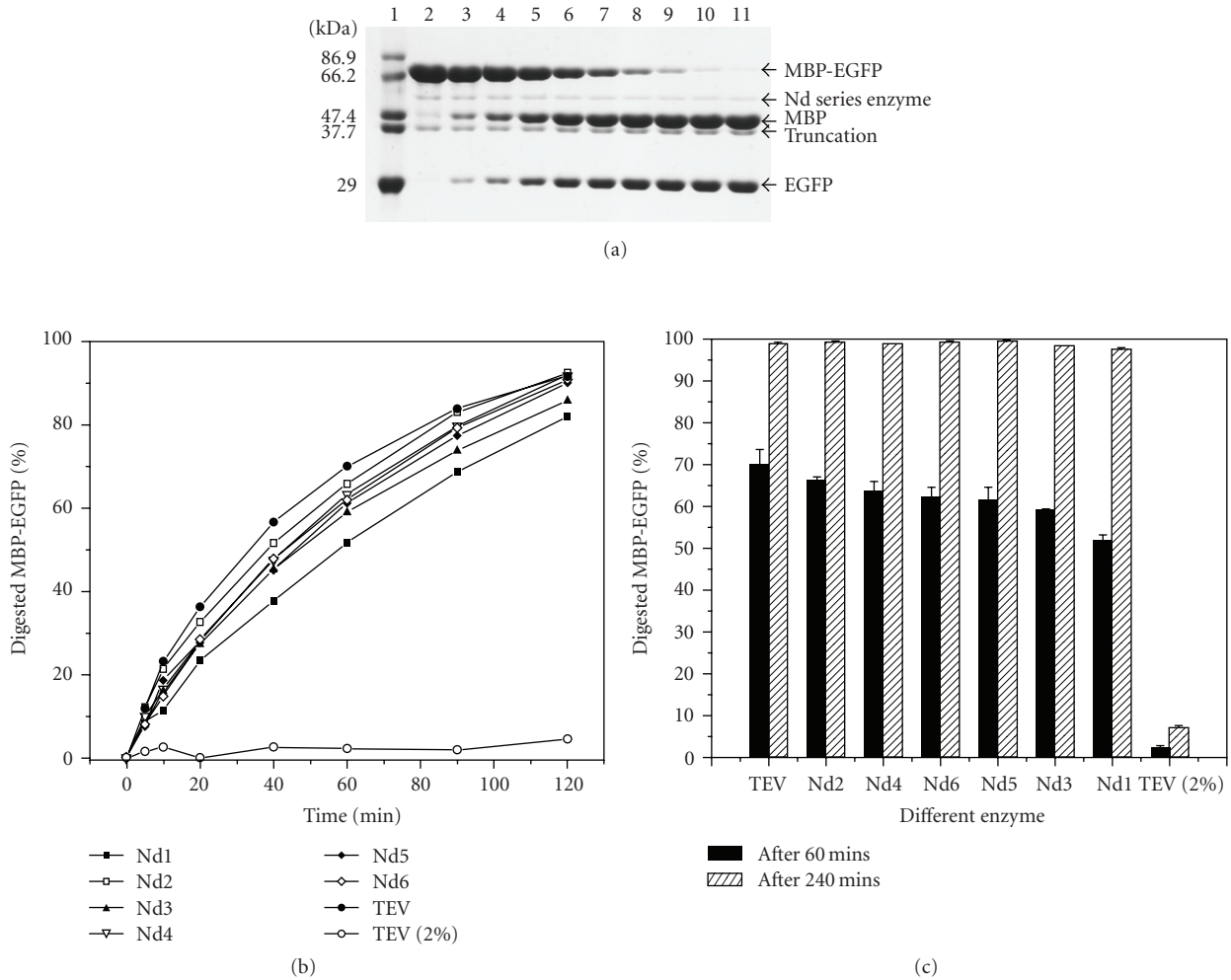


FIGURE 3: SDS-PAGE and time course analysis of catalytic activity on MBP-EGFP of *sfGFP*-TEV-His₆ and TEV-His₆. (a) One representative of SDS-PAGE analysis. 100 μ g MBP-EGFP was incubated with 2 μ g *sfGFP*-TEV-His₆ Nd2. Lane 1 is the protein marker. Lanes 2–11 represent different incubation time (0, 5, 10, 20, 40, 60, 90, 120, 180, 240 minutes, resp.). (b) The time course curve was plotted according to the quantitative analysis of the SDS-PAGE by BandsScan 4.30. Each data point was the average of three independent tests. (c) Bar representation based on time course curve. The black bars represent the percentage of the digested MBP-EGFP after incubation for 60 minutes. The striped bars represent the percentage after incubation for 240 minutes. The ranking of the bar is based on the sorting result of cleavage efficiency after incubation for 60 minutes. The height of the bar is the average of three independent tests with standard error on top of the bar.

production of 400 mg/L culture by optimizing each step in expression, and purification [17]. However, the whole process of expression, purification and characterization of recombinant TEV protease was not visible to naked eye. Our attempts to express recombinant TEV protease fused with commonly used GST, TRX, and NusA tags all failed (data not shown). GST and TRX fused TEV proteases were most in the inclusion body and NusA fusion strategy gave less than 50% full length fusion protein.

In this paper, we introduce a novel method to increase the soluble production of TEV protease by fusing *sfGFP* to TEV protease. The results show that the production of *sfGFP*-TEV-His₆ Nd2 fusion protease reached 320 mg/L culture. Thanks to *sfGFP*'s high folding kinetics, thermodynamic stability, *sfGFP* might work as a platform for the folding of TEV protease to prevent the formation of

inclusion body. Compared with MBP which brings high metabolic burden for the host, *sfGFP* is much smaller and has fluorescence easy for detection. Figure 4 showed that the expression (Figure 4(a)) and purification (Figure 4(b)) procedure of *sfGFP*-tagged fusion protein can be monitored and quantified real-time by the fluorescence emitted from *sfGFP*, thus greatly simplified the procedure of *sfGFP* tagged target proteins expression and purification. We suggest that *sfGFP* could be employed as a colored solubility-enhancing tag for other small proteins with poor soluble production.

Catalytic activity is another important factor to be examined in our work. We constructed 6 variants of *sfGFP*-TEV-His₆ in all. The catalytic tests show that *sfGFP*-TEV-His₆ Nd2 with a linker of only five residues "GSKGP" has the closest catalytic activity to TEV-His₆. After one-hour incubation at 17°C, over 65% MBP-EGFP could be cleaved

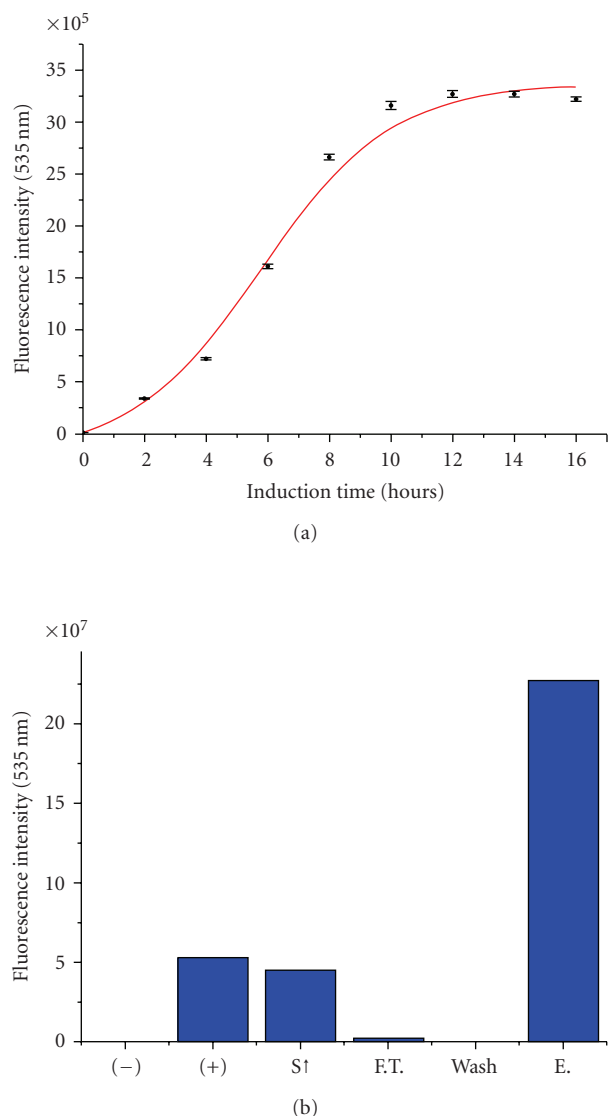


FIGURE 4: The quantification of *sfGFP* fluorescence during *sfGFP-TEV-His₆ Nd2* expression and purification. (a) The fluorescence intensity versus time curve of *sfGFP-TEV-His₆ Nd2* by autoinduction at 19°C. The fluorescence of 100 μ L cultured *E. coli* cells in the autoinduction medium was collected using bottom reading method with 485 nm excitation filter and 535 nm emission filter. The height of the bar is the average of three independent tests with standard error on top of the bar. (b) One representative *sfGFP* fluorescence quantification during Ni-NTA purification. The fluorescence was collected using top reading method with 485 nm excitation filter and 535 nm emission filter. (-): uninduced cell lysate; (+): induced whole cell lysate; S1: supernatant of the sonication; E.T.: flow through fraction from Ni-NTA chromatography; Wash: wash fraction from Ni-NTA chromatography; E.: eluted fraction by imidazole from Ni-NTA chromatography.

by *sfGFP-TEV-His₆ Nd2* while *TEV-His₆* cleaved around 70% substrate (Figure 3). In contrast, *sfGFP-TEV-His₆ Nd1* has the lowest specific activity, which might be explained

by the importance of three residues “KGP” on the correct folding and stability of TEV protease.

When preserved in 4°C for a long time, *TEV-His₆* was not stable and would precipitate and completely lose the catalytic activity within one week (data not shown). However, *sfGFP-TEV-His₆ Nd2* would not precipitate for more than one month and still retained about 60% catalytic activity, which showed much higher stability than original *TEV-His₆*. The *sfGFP* tag not only increased the solubility of the target protein during expression and purification but also increased its stability. Though the increase of effective TEV protease yield of *sfGFP-TEV* was only ~22% (from 140 mg to 171 mg *TEV-His₆* per liter culture), during the long time cleavage experiment, the increased stability of *sfGFP-TEV* significantly outrun the original TEV protease widely used. This feature is vital because structural genomics required large-scale production of tag-free target proteins by TEV protease. Besides, the fluorescence characteristic of *sfGFP* tag provided an accurate, visible, and high-throughput measurement to quantify the fused target protein. The trace existence of *sfGFP* tagged TEV can be sensitive and easily detected by spectrofluorometer. By detecting the *sfGFP* fluorescence intensity, we can also accurately quantify the recombinant *sfGFP-TEV* protease. Like the original TEV protease, the *His₆* tag of *sfGFP-TEV* makes it very easy to remove *sfGFP-TEV* from cleaved target protein by Ni-NTA chromatography after cleavage experiment.

In nature, evolution has shown its power of merging different domains to create a novel enzyme with great property. With rational design, we can also take advantage of available proteins to improve the property of certain enzymes. Our research showed that *sfGFP* tag significantly improved the solubility, expression level, and stability of TEV protease, which is important for the large-scale production of functional TEV protease used in structural genomics research.

Abbreviations

BCA:	Bicinchoninic acid
GFP:	Green fluorescent protein
GST:	Glutathione S-transferase
Ni-NTA:	Nickel-nitrilotriacetic acid
<i>sfGFP</i> :	Superfolder green fluorescent protein
TEV:	Tobacco etch virus protease
TRX:	Thioredoxin.

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