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Engineering soluble tobacco etch virus protease accompanies the loss of stability

Jie Fang, Ling Chen, Beijiu Cheng, Jun Fan *

Key Laboratory of Crop Biology of Anhui Province, Anhui Agricultural University, Hefei 230036, China

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ABSTRACT

Tobacco etch virus protease (TEVp) is a widely used tool enzyme in biological studies. To improve the solubility of recombinant TEVp, three variants, including the double mutant (L56V/S135G), the triple mutant (T17S/N68D/I77V), and the quintuple mutant (T17S/L56V/N68D/I77V/S135G), have been developed, however, with little information on functional stability. Here we investigated the solubility and stability of the three TEVp mutants under different temperature and denaturants, and in *Escherichia coli* with different cultural conditions. The quintuple mutant showed the highest solubility and thermostablity, and the double mutant was most resistant to the denaturants. The double mutant folded best in *E. coli* cells at 37 °C with or without the co-expressed molecular chaperones GroEL, GroES and GrpE. The least soluble wild type TEVp displayed better tolerance to denaturants than the triple and the quintuple mutants. All results demonstrated that TEVp is not engineered to embody the most desirable solubility and stability by the current mutations.

Introduction

Fusion tags are frequently used for improving the solubility and stability of the recombinant proteins, and facilitating the rapid purification by affinity chromatography [1]. Usually, the fused tags are required to be removed, to avoid potential interference with the biological activity or crystallization. This is often achieved by a specific protease that recognizes and cleaves the engineered sequence between the tag and target protein. Tobacco etch virus protease (TEVp)[1] has been widely used as the tool, owing to its stringent sequence specificity. Upon the fusion protein proteolyzed by TEVp, only one amino acid (glycine or serine) is left at N-terminus of the target protein [2].

Production of recombinant TEVp in *Escherichia coli* is hampered by the auto-cleavage and low solubility. To overcome these limitations, one or several amino acid residues in TEVp have been mutated [2]. Replacement of S219 with V219 or N219 conferred the protease with the significant inhibition of auto-proteolysis [3]. Introducing the additional mutations of L56V/S135G into TEVp improves the protein solubility and thermal stability [4]. Another triple mutant (T17S/N68D/I77V) has also been engineered with enhanced solubility [5]. We have combined the five mutations into the TEVp protein and found the quintuple mutant displayed even more solubility than the triple mutant [6].

The functional stability of wild type TEVp has been investigated under various reaction conditions and in the presence of different additives [2]. The optimum temperature for TEVp activity is 37 °C [7]. At relatively high concentrations of denaturants, TEVp retains the greater part of its activity [8]. However, the effects of the reported mutations on the functional stability of TEVp are not known.

Previously, we developed a method for quantitatively measuring the activity of TEVp using a designed fusion protein substrate containing an N-terminally His6-tagged glutathione S-transferase (GST), a TEVp cut and a diaminopropionate ammonia-lyase (DAL). Cleavage of GST-DAL by TEVp enhances the DAL activity thus provides an activity-coupled assay for TEVp [6]. In this study, we analyzed the activities of wild type and three TEVp mutants under various temperatures and the denaturants, and tested the amount and activity of the soluble TEVp in *E. coli* cultured under different conditions. The results revealed that the triple mutations impaired the protease stability. The combined mutations had the synergistic effects on improving protein solubility and thermostability.

Materials and methods

Bacterial strains, plasmids and reagents

E. coli strains DH5α, BL21(DE3) and Rosetta™(DE3), the vectors pET-28a, pRSFDuet-1 and pACYCDuet-1, the FRETWorks

* Corresponding author. Address: Key Laboratory of Crop Science, Anhui Agricultural University, 130, Changjiang West Road, Hefei 230036, Anhui Province, China. Fax: +86 551 65786021.
E-mail address: fanjun@ahau.edu.cn (J. Fan).

[1] *Abbreviations used:* TEVp, tobacco etch virus protease; GST, glutathione S-transferase; GFP, green fluorescent protein; DAL, diaminopropionate ammonia-lyase; Ni-NTA, nickel-nitrilotriacetic acid; 2,4-DNP, 2,4-dinitrophenylhydrazine; PLP, pyridoxal 5′-phosphate; DAP, diaminopropionate.

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S-tag Assay Kit were from Novagen (Madison, WI). Reagents used in the plasmid construction, protein expression, and the site-directed mutagenesis were bought from Takara (Dalian, China). The affinity matrix nickel-nitrilotriacetic acid (Ni–NTA) superflow, urea and guanidine hydrochloride were purchased from Qiagen (Chatsworth, CA). The YM-10 membrane was supplied by Amicon (USA). Pyridoxal 5′-phosphate (PLP) and DL-α,β-diaminopropionate (DL-DAP) were from Sigma (USA). DNA sequencing was performed by Invitrogen (Shanghai, China).

Since all different mutations for improving the protein solubility were independently introduced into the TEVp variant containing S219N or S219V [3–5], we renamed the constructed TEVp mutants, i.e. TEVp^{WT} (S219V, the wild type TEVp in this study), TEVp^{2M} (L56V/S135G), TEVp^{3M} (T17S/N68D/I77V) and TEVp^{5M} (T17S/L56V/N68D/I77V/S135G). Construction of the vector pGST-DAL, other plasmids for independent expression of the N-terminal His6-tagged TEVp^{WT}, TEVp^{3M} and TEVp^{5M} with the C-terminal His6-tag, or S-tag, or green fluorescent protein (GFP) tag, and purification of the double His6-tagged TEVp variants were described previously [6]. The pR-GESP plasmid was generated in our laboratory, in which *E. coli groEL*, *groES* and *grpE* were inserted into pRSFDuet-1. This plasmid contains an artificial operon and every gene was under control of T7 promoter.

Sited-directed mutagenesis and plasmid construction

TEVp^{2M} was constructed using the designed primers with p28-TEVp^{WT} plasmid as template [6]. The resultant plasmid named p28-TEVp^{2M} was subjected to DNA sequencing to confirm the mutations. The mutated gene was excised with Nco I and Xho I, and inserted into the *Nco* I-*Xho* I sites of pACYCDuet-1 to create the vector pA-TEVp^{2M}, or into those of p28EmGFP to yield p28-TEVp^{2M}-GFP plasmid. The plasmids constructed for TEVp purification or TEVp detection by GFP reporter harbored the T7 promoter, a ColE1 origin of replication, and a kanamycin resistance marker. Vectors for TEVp detection by S-tag contained a p15A origin of replication, a chloramphenicol acetyltransferase gene, and a T7 promoter. The plasmid for expressing the molecular chaperones carried a RSF origin of replication, a kanamycin resistance marker and three T7 promoters, and it is compatible with the plasmid for TEVp detection [9].

Purification kinetic analysis of the double His6- tagged TEVp^{2M}

The plasmid p28-TEVp^{2M} was transformed into *E. coli* Rosetta™(DE3) and cultured overnight with kanamycin (50 μg/ml). With dilution 100 fold, the cells were grown in Luria–Bertani (LB) medium until the OD value at 600 nm was up to about 0.7. After induction with 0.5 mM IPTG at 28 °C for 12 h, the cells from 1 l culture were collected, re-suspended in buffer A (50 mM sodium phosphate, 300 mM NaCl and 10 mM imidazole, pH 8.0), sonicated and centrifuged. The supernatant was loaded on a 3 ml Ni–NTA resin packed in a 15 ml column, pre-equilibrated with three column volumes of buffer A, then washed and eluted with three column volumes of 40 and 250 mM imidazole, respectively, in buffer A (pH 8.0). The purified TEVp^{2M} was concentrated by the Ultra-15 centrifugal filter tube equipped with the Ultracel-10 membrane, exchanged with buffer B (20 mM Tris/HCl, 100 mM NaCl, pH 8.0), and analyzed by 15% SDS–PAGE. The gels were stained with coomassie brilliant blue R-250. Protein concentration was determined by Bradford method, using bovine serum albumin as standard. The maximum concentration was determined after the protein sample was ultrafiltrated by centrifugation at 3000*g* for 1 h without causing the obvious change in protein concentration. The concentrated TEVp^{2M} was diluted in buffer B to about 1 mg/ml. Determination of K_m and V_{max} was plotted according to the quantitative analysis from SDS–PAGE. The purified GST-Prx was applied as the substrate, as described previously [6].

Purification of the recombinant DAL

The N-terminally His6-tagged GST-DAL was overexpressed in *E. coli* BL21(DE3) and purified by Ni–NTA resin using the aforementioned procedure. After purification, the concentrated protein was dissolved in buffer B. The GST-DAL proteolyzed by TEVp^{5M} was carried out at 4 °C overnight with the mass ratio of substrate to enzyme 50:1. The cleaved products were loaded on Ni–NTA resin and eluted with buffer A. The purified DAL was concentrated by the Ultra-15 centrifugal filter tube, exchanged with buffer B, and analyzed by 15% SDS–PAGE.

Analysis of DAL activity under different conditions

After 1 h of incubation at various temperature regimes ranging between 30 and 60 °C for 5 °C increment, or 4 h of incubation with urea or guanidine hydrochloride at concentrations of 0.5–2 M with 0.5 M increment, the activity of purified DAL or in the fusion form was assayed. The reaction mixture contains 50 μM PLP, 10 mM DL-DAP and 110 μg of the purified GST-DAL or DAL in a total volume of 100 μl. The assay was started at 37 °C for 5 min and stopped by adding 100 μl of 2 mM HCl containing 0.03% (w/w) 2, 4-DNP. The mixture was incubated for 5 min at 4 °C, and 200 μl of 2 M NaOH was added. Absorbance at 520 nm was recorded in a U-2001 spectrometer (Hitachi, Japan) [6].

Analysis of TEV activity under different conditions

Cleavage of 330 μg of the purified GST-DAL by 66 μg of the TEVp variant was carried out at various temperatures ranging from 30 to 45 °C for 1 h. The activity of DAL with the heat-inactive TEVp^{5M} was also measured as the control and subtracted during the TEVp activity assay. In the presence of urea or guanidine hydrochloride at concentration from 0.5 to 2 M, 100 μg of GST-DAL was incubated with 32 μg of the TEVp variant for 4 h at 25 °C. After reaction, DAL activity was measured. The cleaved products were subjected to SDS–PAGE analysis.

Soluble expression and activity of TEVp variants in E. coli

The plasmid containing gene encoding the TEVp with a N-terminal His6-tag and a C-terminal S-tag was transformed into *E. coli* BL21(DE3). Three colonies were picked randomly and cultured in 50 ml of LB medium independently overnight at 37 °C. When OD_{600} reached about 0.5, the cells were induced by 0.5 mM of IPTG and grown either for 12 h at 28 °C or for 6 h at 37 °C. The cells were collected, re-suspended in buffer B and disrupted by sonication. The supernatant was collected by centrifugation.

The soluble expression level of each TEVp was determined by measuring the fused S-tag amount, according to the Novagen's S•Tag™ System protocol. The reaction mixture contained 20 μl of FRET assay buffer, 2 μl of FRET ArUAA substrate, 5 μl of S-tag grade S-protein, and 153 μl of sterile deionized water. Reactions were initiated by adding 20 μl of the soluble fraction, kept 5 min in the dark, and quenched immediately by the addition of 20 μl of stop solution. The solution was diluted to 2 ml. Each measurement was performed by recording the fluorescence value at excitation and emission wavelengths of 495–530 nm with an F-4500 fluorescence spectrometer (Hitachi, Japan). The amounts of TEVp were represented by the relative fluorescence.

The expression level of all TEVp variants were also detected using GFP reporter. Under the same cultural condition, the cell