An improved variant of tobacco etch virus (TEV) protease that does not need reducing agents

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Abstract

Here we show that a combination of previously suggested mutations for tobacco etch virus (TEV) protease results in a TEV protease mutant that maintains the same catalytic efficiency as previously described mutants but has enhanced stability and solubility. Another advantage of this new variant of TEV protease is that it does not need the inclusion of a reducing agent to maintain its effectiveness, making it easier to generate, store, and use in cleavage reactions compared to previous TEV protease mutants and, in particular, makes it a good choice for cleaving proteins that contain disulfide bonds that would otherwise be altered by the inclusion of a reducing agent. We also provide a straightforward purification protocol for generating this new version of TEV protease.

Keywords: TEV protease, proteolytic cleavage, fusion proteins, tobacco etch virus, protein purification

Statement of Impact for a Broader Audience. Tobacco etch virus (TEV) protease is widely used in laboratories for cleaving fusion proteins. In this work, we introduce a new mutant version of TEV protease that is easier to generate and that has enhanced stability and solubility, properties that will help scientists use this protease to further their research. In addition, this version of TEV protease does not require the presence of reducing agents to maintain its activity.

Introduction

In recombinant protein expression biochemists often generate proteins as fusions with solubility and/or affinity tags that aid in the purification of the desired protein. After purification, these tags typically need to be removed so that they do not affect structural and functional studies of the target protein. To facilitate the removal of tags, a protease cleavage site is frequently added to the fusion protein sequence between the tag and the target protein. The sequence of this cleavage site depends on the protease that will be used; common proteases used for this purpose include Factor Xa (which cleaves after the arginine in the sequences IEGR and IDGR), SUMO protease (which recognizes the small ubiquitin-related modifier (SUMO) tag), and HRV3C protease (which cleaves after the glutamine in the sequence LEVLFQGP).

Arguably the most popular protease for cleaving fusion proteins is tobacco etch virus nuclear-inclusion-a endopeptidase (TEV protease). This cysteine protease has several advantages compared to other proteases. First, it is very specific to its cleavage sequence, so very little off-target activity is observed [1]. Second, it is cost-effective to use as plasmids and protocols for generating it in the laboratory are readily available [2–5]. Third, TEV protease is tolerant of a wide variety of buffer conditions and is resistant to many commonly used protease inhibitors [1, 6–8].

TEV protease has high specificity for the heptapeptide consensus sequence EXϕYϕQθ, where X is any residue, ϕ is any large or medium-sized hydrophobic residue and θ is any small hydrophobic or polar residue [9–11]. Cleavage occurs between the sixth and seventh amino acids of the sequence (i.e., between Q and θ). θ can be a number of residues, but cleavage efficiency is typically greatest when it is G or S, so TEV protease cleavage sites will often have the sequence ENLYFQG or ENLYFQS. Despite the frequent use of G and S, a large number of other amino acids in the θ position can be accommodated by TEV protease; in particular, A and M in the θ position result in cleavage that is almost as efficient as when G or S is used [11].

A number of research groups have used mutagenesis to alter TEV protease to improve its catalytic efficiency, solubility, and stability. For example, the S219V mutation has been shown to increase the stability of the protease as it decreases the rate of autoproteolysis by a factor of approximately 100 compared to wild-type TEV protease [12]. An additional benefit of this mutation is that it doubles the catalytic efficiency relative to wild-type protease. Further mutations based on rational design, such as the combination of L56V and S135G, have been shown to greatly enhance the stability and solubility of TEV protease, allowing it to remain soluble at concentrations 40 times higher than for wild-type protease [13].

Typically, solutions of TEV protease include a reducing agent such as β-mercaptoethanol (BME), dithiothreitol (DTT), or tris(2-carboxyethyl)phosphine (TCEP) to prevent the four cysteines found in the wild-type sequence from becoming oxidized. Although TEV protease is a cysteine protease, it has been shown that it is still able to operate even in the absence of a reducing agent, presumably because the catalytic cysteine (C151) is buried within the active site of the enzyme, thereby greatly reducing its ability to form disulfide bonds with other cysteines [14]. Rather, the need for including a reducing agent when purifying and using TEV protease is because of three other cysteines with varying degrees of exposure to the surface of the protein that, as they slowly oxidize, will result in the formation of inactive oligomers. Consequently, previous groups have proposed additional mutations (C19V, C110V, and C130S) to remove all three non-catalytic cysteines [15, 16], which should remove the need to include a reducing agent during cleavage reactions to keep TEV protease in a monomeric form.

Many other mutations have been proposed that make TEV protease more useful *in vivo* by removing potential glycosylation sites [15, 16] or by making TEV protease more catalytically active in cells [17]. However, as our use of TEV protease has been for *in vitro* applications we have focused on combining the six mutations listed above (C19V, L56V, C110V, C130S, S135G, S219V) into a single variant that we refer to as "TEV Hexa" (Fig. 1). In this paper we show that TEV Hexa has improved solubility and stability compared to a mutant that only has three of these mutations ("TEV

Triple", L56V, S135G, S219V) [13], that it is easy to express with high yields, and that it performs well in the absence of reducing agents. TEV Hexa is available as a plasmid in BL21-RIL *E. coli* cells from Addgene (https://www.addgene.org, plasmid #222868).

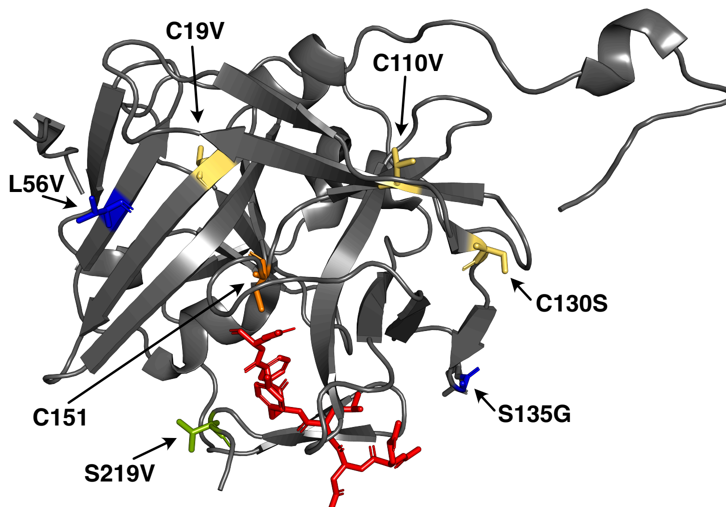

Fig. 1 TEV Hexa incorporates six mutations that increase solubility, stability, and performance in non-reducing conditions. Cartoon representation of TEV protease (gray) bound to substrate (red). The catalytic cysteine (C151) is shown in orange, whereas the three cysteines mutated to other amino acids (C19V, C110V, C130S) are shown in gold. The solubility enhancing mutations (L56V, S135G) are shown in blue and the stability enhancing mutation (S219V) is shown in green. Image rendered using PyMOL [18] based on the X-ray crystal structure (PDB 1LVM) [19].

Results

After expression and two steps of purifications (immobilized metal affinity chromatography and size-exclusion chromatography), our typical yield of TEV Triple from a 250 mL bacterial culture is around 10 mg (corresponding to ≈40 mg of TEV protease per liter of culture), which is similar to the yield reported in a previously reported protocol for generating TEV Triple [4]. For TEV Hexa we consistently get a higher yield of around 14 mg from a 250 mL culture (≈56 mg of TEV protease per liter of culture). Detailed instructions for expressing these proteins are provided in the Supplementary Materials.

To determine the solubility of TEV Triple versus TEV Hexa, we concentrated samples of these proteins in centrifugal concentrators. Both proteins reached a maximum concentration in excess of 500 µM (Fig. 2A). Surprisingly, although the additional mutations in TEV Hexa were not intended to increase the solubility, we found that it had a limiting concentration roughly 10% greater than that for TEV Triple. We hypothesize that the C130S mutation is mainly responsible for this additional solubility as it is the most surface-exposed of the three cysteines that are removed in TEV Hexa and this mutation swaps a hydrophobic amino acid for a polar one. Previous researchers have reported a solubility for TEV Triple [13] that is almost three times greater than what we show in Fig. 2A, a difference that is explained by the inclusion of glycerol in their buffer (200 mM NaCl, 25 mM phosphate pH 8.0, 10% glycerol) to enhance protein solubility, whereas we used a relatively simple buffer (150 mM NaCl, 50 mM phosphate (pH 7.4)). Even without glycerol in any of our buffers, we found the solubility of TEV Triple and TEV Hexa to be sufficiently high to avoid problems with precipitation at all stages of our purification protocol.

To monitor the temporal stability of TEV Triple and TEV Hexa, we placed samples in microcentrifuge tubes and left them at room temperature for several days. Both samples increased in concentration on a short time-scale due to some evaporation of the buffer (Fig. 2B). Over longer time-scales the TEV Triple concentration began to decrease despite the inclusion of 5 mM DTT. DTT has a half-life of around 10 h under the conditions that we used [20] so, as the DTT degraded, TEV Triple began to form disulfide bonds and form aggregates that precipitated out of solution. Other commonly-used reducing agent would also have similar issues with degradation as under these conditions (room temperature, phosphate buffer (pH 7.4)) BME has a ≈10 h half-life [20] and TCEP has a ≈72 h half-life [21]. In addition to having more visible precipitate as the days went on, the TEV Triple sample also formed a gelatinous “skin” at the water/air interface of the sample. We note that this assay only shows total soluble protein and does not demonstrate activity, which we believe (but do not show) should be greatly reduced for TEV Triple compared to TEV Hexa as some of the TEV Triple will be present as smaller oligomers that are still soluble (and therefore measured as part of the concentration) but will no longer be enzymatically active. Experiments that use TEV protease to cleave fusion proteins are typically performed on short enough time-scales (1-2 h at room temperature or overnight at 4°C) that the additional temporal stability of TEV Hexa versus TEV Triple should not be important in the context of a typical cleavage reaction. That said, the additional stability of TEV Hexa is still a useful characteristic as the process of purifying TEV protease takes several hours. For institutions, such as ours, which do not have cold-rooms or refrigerated chromatography instruments, the greater stability of TEV Hexa (and not needing to continously add fresh reducing agents to buffers) makes its production simpler and more reliable to carry out.

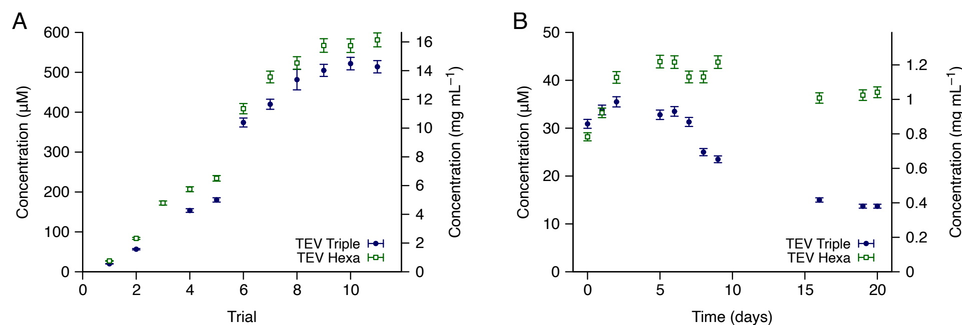

Fig. 2 TEV Hexa is more soluble and temporally stable than TEV Triple. (A) The solubility of TEV Triple and TEV Hexa was determined by repeatedly concentrating the samples and then measuring the concentration (after pelleting out any precipitate). The concentration of TEV Triple plateaued at 514 (±15) µM, whereas TEV Hexa reached a final concentration that was roughly 10% greater (572 (±17) µM). (B) The temporal stability of TEV Triple and TEV Hexa mutants was determined by leaving samples at room temperature in closed microcentrifuge tubes. For both graphs, error bars are set to the standard deviation of triplicate measurements or ±3%, whichever is greater.

Using circular dichroism, we monitored the thermal stability of TEV Hexa and compared it to TEV Triple (Fig. 3A). As has been previously observed for variants of TEV protease in which the surface cysteines are mutated away [16], TEV Hexa is more stable than TEV Triple. For the buffer conditions that we used, the difference in the mid-point temperature for unfolding was 3.5°C. Using tryptophan fluorescence we found that TEV Hexa is more stable with respect to the denaturant guanidinium chloride than TEV Triple, with an unfolding midpoint for TEV Hexa of 1.76 M guanidinium chloride compared to 1.52 M for TEV Triple (Fig. 3B). Like the increase in solubility for TEV Hexa, these modest increases in thermal and chemical stability were surprising as they were not the purpose of the three additional mutations that TEV Hexa has relative to TEV Triple. Most likely, it is the mutations that are closer to the core of the protein (C19V and C110V) that are responsible for the added stability.

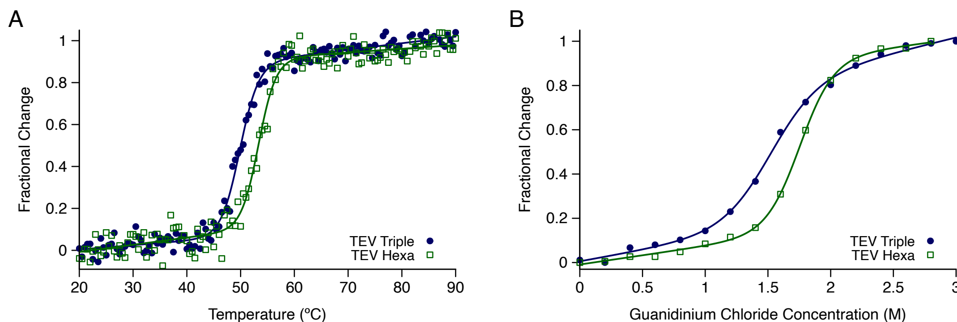

Fig. 3 TEV Hexa is more thermally and chemically stable than TEV Triple. (A) The thermal stabilities of TEV Triple and TEV Hexa were determined by measuring the CD signal at 208 nm as the temperature was increased from 20 to 90°C. After normalization, the data were fit to a two-state model using CDpal. The midpoint temperatures and enthalpies for unfolding were determined to be $T_m = 49.9\ (\pm 0.2)\ ^\circ$C and $\Delta H_m = 490\ (\pm 40)$ kJ mol^{-1} for TEV Triple and $T_m = 53.4\ (\pm 0.2)\ ^\circ$C and $\Delta H_m = 550\ (\pm 50)$ kJ mol^{-1} for TEV Hexa. (B) The chemical stabilities of TEV Triple and TEV Hexa were determined by measuring the tryptophan fluorescence signal as a function of guanidinium chloride concentration. After normalization, the data were fit to a two-state model using CDpal. The denaturant concentration at the midpoint and rate of change in free energy difference were determined to be $C_m = 1.52\ (\pm 0.06)$ M and $m = 13\ (\pm 3)$ kJ mol^{-1} for TEV Triple and $C_m = 1.76\ (\pm 0.02)$ M and $m = 18.4\ (\pm 1.2)$ kJ mol^{-1} for TEV Hexa.

We used gel electrophoresis to compare the enzyme kinetics of TEV Hexa to TEV Triple and also to a version of TEV protease that only has a single mutation (TEV S219V) for the cleavage of a substrate (IC-2C$_{1-96}$) at 30°C; an example of one such gel is provided in Fig. 4A. The band intensities for the cleaved protein (P) and the uncleaved protein (S) were used to determine the concentration of product, [P], at the end of the reaction:

$$[\mathrm{P}] = \left(\frac{P}{S + P}\right)[\mathrm{S}]_0$$

where $[\mathrm{S}]_0$ is the initial concentration of the substrate. The resulting data were fit using nonlinear least squares (nls) in R (4.4.1) [28] to the Michaelis-Menton equation as shown in Fig. 4B:

$$v = \frac{k_{cat}\,[\mathrm{E}]_0\,[\mathrm{S}]_0}{K_m + [\mathrm{S}]_0} \tag{1}$$

where v is the initial rate, k_{cat} is the apparent unimolecular rate constant (the turnover number), $[\mathrm{E}]_0$ is the concentration of enzyme (TEV protease), and K_m is the Michaelis constant. For this analysis, we assumed that $v = \frac{[\mathrm{P}]}{t}$ where t is the total time for the reaction.

As $\frac{[\mathrm{P}]}{t}$ only gives an approximation of the initial rate (especially for small values of $[\mathrm{S}]_0$), the apparent values shown Table 1 from fitting data to Equation 1 are overestimates of k_{cat} and K_m [29]. Consequently, we also analyzed the data using a recently proposed method that does not require true initial rates to get accurate values for k_{cat} and K_m [29]. This method starts with the integrated form of Equation 1:

$$t = \frac{[\mathrm{P}]}{k_{cat}\,[\mathrm{E}]_0} + \frac{K_m}{k_{cat}\,[\mathrm{E}]_0}\ln\left(\frac{[\mathrm{S}]_0}{[\mathrm{S}]_0 - [\mathrm{P}]}\right)$$

and then rearranges this to a form:

$$\frac{[\mathrm{P}]}{t} = -\frac{K_m}{t}\ln\left(\frac{[\mathrm{S}]_0}{[\mathrm{S}]_0 - [\mathrm{P}]}\right) + k_{cat}\,[\mathrm{E}]_0 \tag{2}$$

where plotting $\frac{[\mathrm{P}]}{t}$ vs $\frac{1}{t}\ln\left(\frac{[\mathrm{S}]_0}{[\mathrm{S}]_0-[\mathrm{P}]}\right)$ results in a graph with a slope equal to $-K_m$ and a y-intercept equal to $k_{cat}\,[\mathrm{E}]_0$ as shown in Fig. 4C.

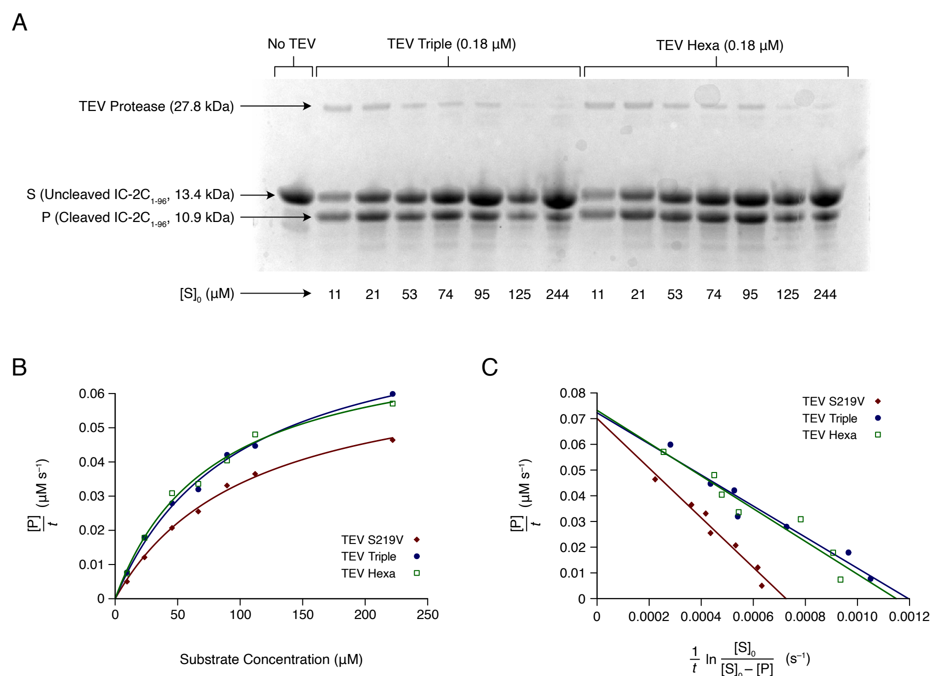

Fig. 4 The rates at which TEV S219V, TEV Triple, and TEV Hexa cleave the substrate IC-2C$_{1-96}$ are comparable. (A) SDS-PAGE results from a kinetics experiment after TEV protease cleavage for 10 min at 30°C. The intensities of the bands (P and S) were used to determine the concentration of product, [P], at the end of the cleavage reaction. To avoid overloading the gel, smaller volumes of gel samples were loaded for cleavage reactions with higher substrate concentrations, resulting in fainter bands for TEV protease on the gel even though the concentration of protease used for all cleavage reactions was kept constant. A 2.5 kDa cleavage product (containing the N-terminal 6×His tag, a short linker, and the TEV cleavage site) was run off the gel. (B) Fitting the results from gel electrophoresis experiments to the Michaelis-Menten model (Equation 1) using nonlinear least squares does not show a significant deviation from the expected hyperbolic relationship even though the rates ($\frac{[P]}{t}$) are not true initial rates. (C) Fitting gel electrophoresis results to Equation 2 using linear least squares better highlights experimental noise and results in more accurate estimations of K_m and k_cat.

Based on fitting kinetics data to Equations 1 and 2, we found that, within error, the kinetic parameters (Table 1) for TEV Triple and TEV Hexa were identical, and very similar to results for a version of TEV protease that only has a single mutation (TEV S219V). We believe that the larger value for K_m (and correspondingly lower catalytic efficiency value) observed for TEV S219V can be attributed to electrostatic repulsion with the substrate; the TEV S219V construct that we used includes a C-terminal polyarginine tag and ≈20% of the IC-2C$_{1-96}$ substrate consists of arginines and lysines. The kinetic similarity between TEV Triple and TEV Hexa is expected as the three additional mutations in TEV Hexa are far from the active site. For older samples of TEV protease and for reactions at lower temperatures (which require much longer cleavage times), TEV Triple cleaved significantly more slowly than TEV Hexa (data not shown), presumably because TEV Triple will slowly oligomerize (and thereby inactivate) if the reducing agent is absent or degrades during the reaction. The results in Table 1 for TEV Triple and TEV Hexa are similar to previously reported kinetic values for TEV S219V [11, 12], with some small differences which we attribute to variations in the substrates and buffer conditions used.

Discussion

Although the various mutations in TEV Hexa have all been previously described [12, 13, 16], this is the first time that these six mutations have been combined in a single plasmid and characterized

Table 1 Kinetic parameters of TEV S219V, TEV Triple, and TEV Hexa for cleaving the substrate IC-2C$_{1-96}$ at 30°C. Using nonlinear least squares to fit estimates of initial rates (rather than true initial rates) to the Michaelis-Menten model (Equation 1) results in overestimates of K_m and k_{cat}, whereas linear least squares fitting to Equation 2 can be used to determine more accurate K_m and k_{cat} values. The values in this table are the averages and standard deviations of the results from fitting multiple individual kinetics experiments.

		k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})
Using Eq 1	TEV S219V	0.28 (±0.02)	0.22 (±0.19)	1.3 (±1.1)
	TEV Triple	0.27 (±0.10)	0.08 (±0.02)	3.3 (±1.6)
	TEV Hexa	0.26 (±0.06)	0.12 (±0.11)	2.2 (±1.9)
Using Eq 2	TEV S219V	0.27 (±0.03)	0.11 (±0.02)	2.6 (±0.6)
	TEV Triple	0.26 (±0.05)	0.045 (±0.014)	6 (±2)
	TEV Hexa	0.26 (±0.06)	0.044 (±0.011)	6 (±2)

to demonstrate similar or improved behavior compared to previously described mutants. Although the enhanced behavior of TEV Hexa in terms of stability in the absence of reducing agents was expected, we were surprised to find that it was also slightly more soluble, thermally stable, and chemically stable, than previously characterized mutants. The ability to use TEV Hexa without including a reducing agent (such as BME, DTT, or TCEP) is particularly useful when cleaving proteins with disulfide-bonds that might otherwise be degraded by the reducing agent. Although it is possible to use "regular" TEV protease with proteins that contain disulfide-bonds (for example, for cleaving inhibitor cystine knot peptides [14]) by using a redox buffer containing reduced (GSH) and oxidized (GSSG) glutathione this can still require some optimization to prevent scrambling of disulfide bonds.

We note that a plasmid with similar TEV protease mutations has been recently made available (pET28-MBP-super TEV protease, Addgene plasmid #171782) [22]. However, this variant of TEV protease differs in that it has a S219D mutation rather than the S219V mutation that is included in TEV Hexa. The S219V mutation has been shown to reduce autolysis by a factor of 10 relative to the S219D mutation (and a factor of 100 compared to wild-type TEV protease). In addition, the S219V mutation has a catalytic efficiency (k_{cat}/K_m) that is roughly two-fold higher than both wild-type and S219D TEV protease due to both a smaller K_m value and a larger k_{cat} value [12]. Our TEV Hexa construct also retains a TEV protease cleavage site between the MBP tag and the protease. Consequently, the MBP tag is cleaved during expression and removed during IMAC purification, resulting in a smaller protease that can diffuse more quickly in cleavage reactions.

Another recently released plasmid for TEV protease (pET29b-10xHis-Super TEV, Addgene plasmid #193833) [5] includes three mutations (L56V, S135G, S219V) present in TEV Triple and TEV Hexa, as well as six additional mutations (T17S, N68D, I77V, I138T, S153N, T180A). Three of these additional mutations (T17S, N68D, I77V) are believed to increase the solubility of TEV protease [23]. However, the paper that identified those mutations did not directly measure solubility; rather, the yield of TEV protease after purification was used as a stand-in for solubility. As the yield observed [5] is similar to the yield reported for TEV Triple [4] and for TEV Hexa (this paper), it is unclear if there is any net advantage to including both the T17S/N68D/I77V and the L56V/S135G mutations in a single variant of TEV protease. It could be that either of these sets of mutations results in sufficiently high solubility that solubility is no longer a limiting factor in the expression and usage of TEV protease. The combination of the other three mutations (I138T, S153N, T180A) have been shown to increase TEV protease activity by roughly two-fold by increasing the binding affinity for the substrate (i.e., decreasing K_m) [17]. However, the S153N mutation borders the active site and results in some small changes to the specificity. For example, the P1 position (the first amino acid in the N-terminal direction from the site of cleavage) is almost always a glutamine for wild-type

TEV protease, but when the S153N mutation is included the specificity at this position is reduced [17]. When using TEV protease to cleave folded proteins these subtle changes in specificity are most likely not a concern as other potential cleavage sites (which will in any case be very rare) will typically be inaccessible to the protease. However, most of our current research involves working with intrinsically disordered proteins where the specificity of TEV protease is vitally important and where the speed of the cleavage reaction is not a limiting factor. For this reason, in designing TEV Hexa we deliberately avoided adding mutations that are near the active site to reduce the chance of inadvertently changing the binding specificity. Finally, this nine-mutant variant does not include the mutations that remove unneeded cysteines so, unlike TEV Hexa, it has the same need as TEV Triple for the inclusion of reducing agents during purification and cleavage reactions to maintain solubility and catalytic activity.

Conclusion

Previously described TEV protease mutants have already greatly improved it for use in biochemical applications. Although the modest improvements in stability and solubility that we show for our TEV Hexa mutant will probably not revolutionize the use of TEV protease in the laboratory, the benefit of not needing to continuously add freshly-prepared reducing agents at every stage of TEV protease purification, for storage, and for cleavage reactions, makes TEV Hexa much easier to keep fully active compared to other TEV protease mutants. In addition, TEV Hexa is well suited to use with proteins that contain disulfide bonds as cleavage can be carried out without needing to add a redox buffer or reducing agents. We hope that the conveniences of TEV Hexa's extra stability and easier handling will appeal to scientists who routinely use TEV protease for cleaving fusion proteins.

Materials and Methods

Site-Directed Mutagenesis

The plasmid for the "TEV Triple" construct containing the L56V, S135G, and S219V mutations [4] was a gift from David Waugh (Addgene plasmid #92414). This pMAL-c2 derived plasmid expresses TEV protease with an N-terminal maltose binding protein (MBP) fusion tag followed by a TEV protease cleavage site and a heptahistidine (7×His) tag. Back-to-back primers to add the C19V, C110V, and C130S mutations were designed using NEBaseChanger [24] and synthesized by Integrated DNA Technologies (Coralville, IA). Mutations were added sequentially using the Q5 site-directed mutagenesis kit (New England Biolabs, Ipswich, MA) and confirmed by Sanger sequencing of the resulting plasmids. The final plasmid with all six mutations ("TEV Hexa") was deposited with Addgene (https://www.addgene.org, plasmid #222868).

Protein Expression and Purification

For protein expression, plasmids containing the TEV Triple and TEV Hexa constructs were used to transform BL21-CodonPlus-RIL *Escherichia coli* cells. Colonies of transformants were grown in LB media and used to generate glycerol stocks that were then frozen at −80°C.

Cultures of 250 mL of ZYM-5052 media [25] with 100 µg L^{-1} carbenicillin and 30 µg L^{-1} chloramphenicol were inoculated using scrapings from frozen glycerol stocks and grown for 60 h at 25°C in 2 L baffled flasks. The cultures were harvested by centrifugation and the resulting cell pellets were resuspended in 25 mL of ice-cold lysis buffer (250 mM NaCl, 20 mM imidazole, 20 mm phosphate (pH 7.4), 2 mM DTT, 1 mM AEBSF, 50 µM bestatin, 15 µM E-64, 15 µM pepstatin, 8.5 µM phosphoramidon). After lysis by ultrasonication, lysates were clarified by high-speed centrifugation (42,000×rcf for 30 min at 4°C).

The resulting supernatant was then purified by immobilized metal affinity chromatography (IMAC) using a Cytiva (Marlborough MA) Äktaprime plus liquid chromatography system with a

5 mL Cytiva HisTrap column. For IMAC, the binding buffer contained 500 mM NaCl, 50 mM phosphate (pH 7.4), and 20 mM imidazole. Protein was eluted using an imidazole gradient that increased to 500 mM. Elution fractions containing protein (as determined by monitoring the A_{280}) were combined, concentrated to less than 2 mL using 10 kDa Amicon Ultra (MilliporeSigma, Burlington, MA) centrifugal filters, and then purified by size-exclusion chromatography (SEC) using an Äktaprime plus liquid chromatography system with a Cytiva HiPrep 16/60 Sephacryl S100 column and a 150 mM NaCl/50 mM phosphate (pH 7.4) running buffer ("SEC buffer").

SEC fractions containing protein were pooled and quantified by measuring the A_{280} using a Thermo Fisher (Waltham, MA) NanoDrop 2000c spectrophotometer (the TEV Triple and TEV Hexa constructs have a molar absorptivity at 280 nm of 31,970 L mol^{-1} cm^{-1}). Most assays were carried out with freshly prepared TEV protease. For long-term storage, TEV protease samples were diluted with glycerol to achieve a 50% glycerol concentration and a TEV protease concentration of 25 μM (0.69 mg mL^{-1}) and then stored at −80°C.

Solubility Assay

Samples of TEV protease in SEC buffer were repeatedly concentrated using 10 kDa Amicon Ultra centrifugal filters. After each round of concentration, the sample was removed from the filter and placed in a microcentrifuge tube. After centrifugation at 22,000×rcf for 1 min to pellet aggregated protein, the A_{280} of the supernatant was measured using a NanoDrop 2000c spectrophotometer after which the supernatant was returned to the centrifugal filter for further concentration.

Temporal Stability Assay

Samples of ≈30 μM TEV protease in SEC buffer were left to incubate at room temperature for two weeks in closed microcentrifuge tubes. Every few days, the A_{280} of the supernatant was measured using a NanoDrop 2000c spectrophotometer after centrifugation at 22,000×rcf for 1 min to pellet any particulates/precipitates.

Thermal Stability Assay

The thermal stability of the TEV protease mutants in SEC buffer at a concentration of 10 μM was studied using circular dichroism (CD) spectrometry. To do this, the CD signal at 208 nm for the proteases was monitored over a temperature range of 20–90°C (heating at 2°C min^{-1}) using a JASCO (Easton, MD) J-810 CD spectrometer and a 1 mm path length quartz glass cuvette. The CD signal was then normalized and fit to a two-state model ($N \rightleftharpoons D$) using CDpal [26] with the change in heat capacity between the two states (ΔC_p) set to zero.

Equilibrium Unfolding Assay

TEV protease samples at a concentration of 0.95 μM in SEC buffer with between 0 and 3 M guanidinium chloride were allowed to equilibrate for 2 h at 30°C, after which fluorescence emission spectra were acquired over a range of 290 to 500 nm using an Agilent (Santa Clara, CA) Cary Eclipse fluorescence spectrometer with a 1 cm path length quartz glass cuvette. The spectra were acquired using an excitation wavelength of 280 nm, a scan speed of 120 nm min^{-1}, and slit widths set to 5 nm. The center of the tryptophan fluorescence peak was determined by calculating the center of spectral mass (COSM):

$$\mathrm{COSM} = \frac{\sum \lambda_i F(\lambda_i)}{\sum F(\lambda_i)}$$

where $F(\lambda_i)$ is the fluorescence emission intensity at wavelength λ_i. The COSM values as a function of guanidinium chloride concentration were then normalized and fit to a two-state ($N \rightleftharpoons D$) model using CDpal [26].

Enzyme Kinetics by Gel Electrophoresis

Enzyme kinetics were determined by mixing 10 μL of 0.36–0.50 μM TEV protease (final concentration 0.18–0.25 μM) with 10 μL of 15–500 μM substrate (final concentration 7.5–250 μM) in SEC buffer. For comparison, we also ran kinetics trials with a variant of TEV protease that only has the S219V mutation [12], which was a gift from David Waugh (Addgene plasmid #8827). In addition to lacking the additional mutations of TEV Triple and TEV Hexa, TEV S219V also differed in having a C-terminal polyarginine tag. The substrate, IC-2C$_{1-96}$, is an N-terminal fragment of dynein intermediate chain with an N-terminal 6×His tag followed by short flexible linker and a TEV protease cleavage site. This construct was designed to result in a native protein sequence after TEV cleavage, so the sequence for the cleavage site was ENLYFQM rather than the more usual ENLYFQG or ENLYFQS sequences used for TEV cleavage. Each mixture of substrate and protease was allowed to react for 10 min at 30°C before quenching it by adding 10 μL of 3× loading buffer (62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% glycerol, 0.01% (w/v) bromophenol blue, 40 μM DTT) and heating at 90°C for 5 min. The resulting samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using GenScript (Piscataway, NJ) SurePAGE™ Bis-Tris 8-16% gels. After electrophoresis, the gels were stained using Bulldog Bio (Portsmouth, NH) AcquaStain and then imaged, baseline corrected using the rolling disk method, and quantified using a Bio-Rad (Hercules, CA) Gel Doc XR+ system with Bio-Rad Image Lab 6.1 software.

Accessibility Statement. TEV "Hexa" (C19V, L56V, C110V, C130S, S135G, S219V) is available as a plasmid in BL21-RIL *E. coli* cells from Addgene (https://www.addgene.org, plasmid #222868).

Supplementary Material Description. The supplementary material consists of a detailed protocol for producing the TEV Triple and TEV Hexa mutants of TEV protease.

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Supplementary Material

Protocol for Producing TEV Protease

The following protocol provides reasonable yields of TEV protease with minimal effort, and can easily be adapted to whatever combination of equipment and methods are available in your laboratory. This is not an extensively optimized protocol, so you should feel free to experiment with different conditions if those are more convenient for you as they may lead to equally good (or possibly better) yields.

1. Order the plasmid for the TEV Hexa mutant from Addgene (plasmid #222868). TEV Triple is also available from AddGene (plasmid #92414) but has no advantages compared to TEV Hexa. Addgene ships these plasmids as bacterial stabs in BL21-CodonPlus-RIL *E. coli* cells.
   - Once received, streak and isolate bacteria from the stab culture onto an LB agar plate (See https://www.addgene.org/protocols/streak-plate/ for directions).
   - The plate should include carbenicillin (or ampicillin) at 100 μg mL^{-1} to select for bacteria carrying the plasmid with the TEV protease gene. In addition, to maintain the additional plasmid (pRIL) in the BL21-CodonPlus-RIL cells with the genes for tRNAs for rare codons, you should also include chloramphenicol (30 μg mL^{-1}).
   - As the gene for TEV protease is controlled by a tac promoter rather than a T7 promoter, the use of a DE3 *E. coli* strain is not necessary for protein expression.
   - We have also tried using regular BL21 cells rather than CodonPlus-RIL cells; when using regular BL21 cells expression levels of TEV protease drop by roughly one-third.
2. For long-term storage of cells for protein expression, it is advisable to generate glycerol stocks from single colonies from plates of transformants and then to store the glycerol stocks at −80°C (See https://www.addgene.org/protocols/create-glycerol-stock/ for directions).
3. Inoculate a 250 mL culture of ZYM-5052 media [25] supplemented with 100 μg L^{-1} carbenicillin and 30 μg L^{-1} chloramphenicol and grow for ≈60 h at 25°C in a 2 L baffled flask.
   - A single colony from a plate of transformants, or a scraping from a glycerol stock stored at −80°C, can be used to inoculate the culture.
   - For our incubator, shaking at 125 rpm provides a good amount of aeration for 2 L baffled flasks.
   - We find using autoinducing media and a long growth time at a relatively low temperature convenient because we can set up a culture on a Friday afternoon and come back to harvest it Monday morning.
   - Ampicillin can be used instead of carbenicillin. However, we prefer to use carbenicillin as it is more stable so it is better suited for maintaining antibiotic selection for long incubation times.
4. Harvest the cultures by centrifugation (≈3000×rcf for ≈20 min) and then resuspend the resulting cell pellets in 25 mL of ice-cold lysis buffer (250 mM NaCl, 20 mM imidazole, 20 mm phosphate (pH 7.4), 2 mM DTT, and protease inhibitor cocktail).

- Our lysis buffer includes a protease inhibitor cocktail of 1 mM AEBSF, 50 μM bestatin, 15 μM E-64, 15 μM pepstatin, and 8.5 μM phosphoramidon. However, this is not needed if samples are processed quickly and kept on ice whenever possible. Alternatively, other protease inhibitor cocktails can be used (such as Roche cOmplete™ protease inhibitor cocktail), as long as inhibitors that react with cysteine (such as N-ethylmaleimide (NEM) and iodoacetamide) are not included [6]. Interestingly, E-64 (an irreversible inhibitor of cysteine proteases) does not inhibit the activity of TEV protease [6].
- DTT is included as a reducing agent when we generate TEV Triple to prevent intermolecular disulfide bonds from forming. It should not be needed for TEV Hexa but it does not hurt to include it.
- Many other procedures include glycerol (typically at 10%) in buffers used with TEV protease to increase its solubility (and therefore reduce the chance of accidental precipitation). We find including glycerol is not necessary for the highly soluble TEV Triple and TEV Hexa mutants.

5. After lysis by ultrasonication, clarify the lysates by high-speed centrifugation (42,000×rcf for 30 min at 4°C).
6. Purify the lysates by immobilized metal affinity chromatography (IMAC).
   - We filter our clarified lysates using Phenomenex glass fiber/cellulose acetate syringe filters prior to IMAC to increase the longevity of our IMAC columns.
   - We keep our samples on ice or at 4°C when possible during purification. However, our chromatography instruments and the buffers used with them are at room temperature. As long as samples are processed quickly, it is not critical to keep TEV protease samples cold at every step of the purification protocol.
   - For IMAC, we use a Cytiva (Marlborough MA) Äktaprime plus liquid chromatography system with a 5 mL Cytiva HisTrap column (which theoretically is able to bind in excess of 200 mg of His-tagged protein). Before applying the sample, the column is equilibrated with "binding buffer" (500 mM NaCl, 50 mM phosphate (pH 7.4), 20 mM imidazole). After applying the sample, we wash the column with more binding buffer and then elute His-tagged proteins using a steadily increasing gradient of "elution buffer" (500 mM NaCl, 50 mM phosphate (pH 7.4), 500 mM imidazole).
   - If using a "Superloop", it is best to clean out the loop after purifying TEV protease before purifying regular His-tagged proteins. The residual amount of material that can persist in the Superloop can result in unwanted on-column cleavage. Likewise, it is a good idea to reserve an IMAC column for TEV protease purifications as residual TEV protease bound to the IMAC column may cause problems with on-column cleavage.
   - We have also successfully used Cytiva His GraviTrap columns (which theoretically are able to bind in excess of 40 mg of His-tagged protein) for purification. For purification with a GraviTrap column, we recommend using a wash buffer with an intermediate concentration of imidazole (50-75 mM) after applying the sample to the column to remove weakly-binding contaminates before releasing the His-tagged protein from the column with elution buffer containing 500 mM imidazole.
7. Combine elution fractions containing protein as determined by monitoring the A_{280}.
   - We sometimes see two peaks in our chromatogram from IMAC. The first peak (eluting at a lower imidazole concentration) corresponds to metal-binding bacterial proteins, whereas the second peak (eluting at a higher concentration of imidazole) corresponds to 7×His-tagged TEV protease. We find that 7×His-tagged TEV protease elutes when the imidazole concentration in the gradient elution exceeds ≈300 mM.
   - If purifying TEV Triple, it is a good idea to add DTT to a final concentration of 2 mM to the pooled IMAC fractions so that disulfide bonds do not begin to form.

- We sometimes will skip the additional purification detailed in the next three steps and use TEV protease directly after IMAC purification. Although there will be some impurities in the TEV protease sample at this point, TEV protease is usually added in a 1:50 or 1:100 molar ratio when it is used to cleave substrate. Consequently, whatever impurities there are in the TEV protease sample will be further diluted in the cleavage reaction sample (and should be removed by whatever additional post-cleavage purification steps are carried out to separate fusion tags from target proteins).

8. If carrying out size-exclusion chromatography (SEC), concentrate the combined IMAC elution fractions to a volume appropriate for your loading loop.
   - Our SEC system has a 5 mL loading loop, so we typically concentrate our samples to a bit less than half of that (≈2 mL) using 10 kDa Amicon Ultra centrifugal filters.
   - We filter our concentrated TEV protease using 0.2 µm syringe filters prior to SEC to increase the longevity of our SEC columns.
   - When concentrating the TEV protease prior to SEC, we typically reach concentrations of around 300 µM (≈10 mg mL^{-1}). This is approximately half of the maximum solubility of TEV Triple and TEV Hexa and we have had no problems with precipitation at this step. That said, if you are generating TEV protease on a larger scale, it may be necessary to add 10% glycerol at this point to increase TEV protease solubility, to use a larger sample loading loop, and/or to carry out multiple SEC runs, to avoid over-concentrating/precipitating your protein.
9. Carry out SEC to further purify TEV protease.
   - For SEC we use an Äktaprime plus liquid chromatography system with a Cytiva HiPrep 16/60 Sephacryl S100 column and a 150 mM NaCl, 50 mM phosphate (pH 7.4) running buffer ("SEC buffer"). With this column, we find that fractions corresponding to 58–73 mL into the SEC run contain TEV protease.
10. Pool SEC fractions containing protein and quantify the concentration of TEV protease by measuring the A_{280}.
    - The TEV Triple and TEV Hexa constructs both have molar absorptivities at 280 nm of 31,970 L mol^{-1} cm^{-1}.
    - If needed, we further concentrate the SEC-purified TEV protease using 10 kDa Amicon Ultra centrifugal filters.
    - If purifying TEV Triple, it is a good idea to add DTT to a final concentration of 2 mM to the pooled SEC fractions so that disulfide bonds do not begin to form.
11. TEV Triple and Hexa can be stored in SEC buffer at 4°C for a few days if they will be promptly used. For long-term storage, they should be diluted with glycerol to achieve a 50% glycerol concentration and then stored at −80°C.
    - We usually aim for a final TEV protease concentration of 25 µM (0.87 mg mL^{-1}) for our frozen stocks of TEV protease. Although TEV Triple and TEV Hexa can be stored at much higher concentrations, we find this concentration convenient for our use.
12. Our typical yield of TEV Hexa from this protocol using a 250 mL culture is around 14 mg (corresponding to ≈56 mg L^{-1} of culture). This protocol can easily be scaled up if more protease is needed.

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