Chapter 19

Expression and Purification of Soluble His_6-Tagged TEV Protease

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Summary

This chapter describes a simple method for overproducing a soluble form of the tobacco etch virus (TEV) protease in *Escherichia coli* and purifying it to homogeneity so that it may be used as a reagent for removing affinity tags from recombinant proteins by site-specific endoproteolysis. The protease is initially produced as a fusion to the C-terminus of *E. coli* maltose binding protein (MBP), which causes it to accumulate in a soluble and active form rather than in inclusion bodies. The fusion protein subsequently cleaves itself *in vivo* to remove the MBP moiety, yielding a soluble TEV protease catalytic domain with an N-terminal polyhistidine tag. The His-tagged TEV protease can be purified in two steps using immobilized metal affinity chromatography (IMAC) followed by gel filtration. An S219V mutation in the protease reduces its rate of autolysis by approximately 100-fold and also gives rise to an enzyme with greater catalytic efficiency than the wild-type protease.

Key words: Maltose-binding protein; MBP; Immobilized metal affinity chromatography; IMAC; His-tag; Affinity tag; Affinity chromatography; Tobacco etch virus protease; TEV protease; Fusion protein

19.1. Introduction

The use of genetically engineered fusion tags has become a widespread practice in the production of recombinant proteins for various applications. Although originally designed to facilitate the detection and purification of proteins, subsequently it has become clear that some tags can also increase the yield of their fusion partners, protect them from intracellular proteolysis, enhance their solubility and even facilitate their folding *(1)*. However, all tags, whether large or small, have the potential to interfere with the

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biological activity of a protein and may impede its crystallization (e.g., *2–5)*. For this reason, it is generally advisable to remove the tag(s) at some stage.

Although both chemical and enzymatic reagents have been used to remove tags from recombinant proteins *(6)*, only proteases exhibit enough specificity to be generally useful for this purpose. Traditionally, the enzymes that have been used most commonly to cleave fusion proteins at designed sites are Factor Xa, enterokinase (enteropeptidase) and thrombin. However, all of these proteases have frequently been observed to cleave proteins at locations other than the designed target site *(7, 8)*.

Recently it has become clear that certain viral proteases, such as that encoded by the tobacco etch virus (TEV), have much greater stringency *(9)*, making them particularly useful for this application. TEV protease recognizes the amino acid sequence ENLYFQ/G with high efficiency and cleaves between Q and G. Hence, in contrast to Factor Xa and enterokinase, which ostensibly have no specificity for the P1′ residue in their respective recognition sites, TEV protease typically leaves a single non-native glycine residue on the N-terminus of a recombinant protein when it is used to remove an N-terminal fusion tag. It should be noted, however, that the P1′ specificity of TEV protease is relatively relaxed and many different residues can be substituted for glycine in the P1′ position of its recognition site with little or no impact on the efficiency of cleavage *(10)*, making it possible to produce many proteins with no non-native residues appended to their N-terminus.

Unfortunately, TEV protease is poorly soluble when it is overproduced in *Escherichia coli (11, 12)*. Yet, we have found that this problem can be overcome by producing the enzyme in the form of a MBP fusion protein that cleaves itself *in vivo* to generate a TEV protease catalytic domain that is highly soluble and active. Fusion to MBP somehow enables the enzyme to fold into its native conformation and avoid accumulating as insoluble aggregates *(11)*. Another problem with TEV protease is that it cleaves itself at a specific site, giving rise to a truncated enzyme with greatly diminished activity *(13)*, but this has been overcome by the introduction of a mutation (S219V) that renders the enzyme virtually impervious to autoinactivation and also increases its catalytic activity by approximately twofold *(14)*.

Here, we describe a method for the large-scale production of S219V mutant TEV protease in *E. coli* and its purification to homogeneity. The presence of a polyhistidine (His6-tag) on the N-terminus of the protease facilitates not only its purification but also its separation from the digestion products of a His-tagged fusion protein *(15)*. Although not discussed here, the polyarginine tag on the C-terminus of the catalytic domain can be used in a similar manner.

19.2. Materials

19.2.1. Overproduction of His_6-TEV(S219V)-Arg_5 Protease in E. coli

1. A glycerol stock of *E. coli* BL21(DE3) CodonPlus-RIL cells (Stratagene, La Jolla, CA, USA) containing the TEV protease expression vector pRK793 (*see* **Note 1**).
2. LB broth and LB agar plates containing 100 µg/ml ampicillin (for pRK793 selection) and 30 µg/ml chlorampenicol (for pRIL selection). For LB broth, add 10 g bacto tryptone, 5 g bacto yeast extract and 5 g NaCl to 1 l of H_2O and sterilize by autoclaving (121°C, 15 psi, 20 min, slow exhaust). Let cool to room temperature and add 1 ml of sterile 100 mg/ml ampicillin and 1 ml of 30 mg/ml chloramphenicol. Prepare a 100 mg/ml ampicillin solution by mixing 10 g of ampicillin, sodium salt, ultra with H_2O to a final volume of 100 ml. Filter sterilize and store at –20°C. Prepare a 30 mg/ml chloramphenicol solution by mixing 3 g of chloramphenicol with absolute ethanol to a final volume of 100 ml. Store at –20°C. Sterile glucose can be added to the LB-antibiotic broth to a concentration of 0.2% to increase the bacteria biomass. Prepare a stock solution of 20% (w/v) of D(+)-glucose monohydrate by mixing 100 g of D(+)-glucose monohydrate with H_2O to a final volume of 500 ml. Filter sterilize and store at room temperature. Add 10 ml 20% sterile glucose per 1 l of LB broth to achieve a 0.2% concentration. For LB agar add 12 g of Bacto agar per 1 l of LB broth before autoclaving (as above). To prepare plates, allow the 1 l of LB-agar mixture to cool until the flask or bottle can be held in hands without burning (approximately 50–60°C). Add 1 ml of 100 mg/ml sterile ampicillin stock solution (*see* above) and 1 ml of 30 mg/ml chloramphenicol stock solution (*see* above) to the LB-agar, mix by gentle swirling and pour or pipet ca. 30 ml into sterile 100 × 15 mm Petri dishes. Let plates cool to room temperature and store at 4°C.
3. Isopropyl-β-D-thiogalactopyranoside (IPTG), analytical. Prepare a fresh 200 mM stock solution by mixing 477 mg of IPTG with H_2O to a final volume of 10 ml. Five ml of 200 mM IPTG is required per 1 l of culture to achieve a final concentration of 1 mM.
4. A 500 ml and several 4 l baffle-bottom flasks. Sterilize by autoclaving (as above, fast exhaust).
5. An autoclave with fast and slow exhaust setting.
6. A temperature-controlled shaker/incubator that can accommodate 500 ml and 4 l flasks and can be set at either 30 or 37°C.
7. A high speed centrifuge (e.g., Sorvall refrigerated centrifuge).
8. A spectrophotometer and cuvette that can measure absorbance at 600 nm.

19.2.2. Purification of His_6-TEV(S219V)-Arg_5 Protease

1. Cell lysis/IMAC equilibration buffer: 50 mM sodium phosphate (pH 8.0), 200 mM NaCl, 10% glycerol, 25 mM imidazole. Prepare 2 l of buffer by mixing 14.2 g sodium phosphate dibasic, 23.38 g NaCl, 200 ml glycerol and 3.41 g imidazole with H_2O to a volume of 1980 ml. Adjust the pH to 8 using concentrated hydrochloric acid. Adjust the volume to 2 l with H_2O and check the pH. Adjust if necessary. Filter through a 0.22 µm polyethersulfone membrane (Corning Incorporated, Corning, NY, USA, or the equivalent) and store at 4°C.
2. A mechanical device to disrupt E. coli cells (e.g., a sonicator, french press, or cell homogenizer) (see Note 2)
3. A solution of 5% (w/v) polyetheleneimine, pH 8. Mix 50 ml of 50% (w/v) polyethylenimine with H_2O to a volume of 450 ml. Adjust the pH to 8 with concentrated HCl and let to cool to room temperature. Adjust the volume to 500 ml with H_2O and check the pH. Adjust if necessary. Filter through a 0.22 µm polyethersulfone filtration unit (Corning Incorporated, Corning, NY, USA, or the equivalent). The solution is stable for at least 3 years when stored at 4°C.
4. A spectrophotometer and cuvette that can measure absorbance at 280 nm.
5. ÄKTA Explorer chromatography system (Amersham Biosciences, Piscataway, NJ, USA), or the equivalent.
6. Ni-NTA Superflow resin (Qiagen Incorporated, Valencia, CA, USA).
7. Column XK 26/10 (Amersham Biosciences, Piscataway, NJ, USA).
8. IMAC equilibration and elution buffers are: (a) 50 mM sodium phosphate (pH 8.0), 200 mM NaCl, 10% glycerol, 25 mM imidazole, and (b) 50 mM sodium phosphate (pH 8.0), 200 mM NaCl, 10% glycerol, 250 mM imidazole, respectively. For preparation of IMAC (Ni-NTA Superflow) equilibration buffer (see Step 1 in Section 19.2.2). Prepare 1 l of elution buffer by mixing 7.1 g sodium phosphate dibasic, 11.69 g NaCl, 100 ml glycerol and 17.02 g imidazole, with H_2O to a volume of 950 ml. Adjust the pH to 8 using concentrated hydrochloric acid. Adjust the volume to 1 l with H_2O, let to cool to room temperature and check the pH. Adjust if necessary. Filter through a 0.22 µm polyethersulfone membrane (Corning Incorporated, Corning, NY, USA, or the equivalent) and store at 4°C.
9. A 0.5 M ethylenediaminetetraacetic acid (EDTA), pH 8 stock solution.
10. A 1 M stock solution of 1,4-dithio-DL-threitol (DTT). Prepare 10 ml by mixing 1.55 g of DTT with H_2O to a final

volume of 10 ml. Place solution on ice. Use immediately or store at –20°C.

11. Polyethersulfone filtration unit (0.22 and 0.45 µm, Corning Incorporated, Corning, NY, USA, or the equivalent).
12. An Amicon Stirred Ultrafiltration Cell concentrator and YM10 ultrafiltration membranes (Millipore Corporation, Bedford, MA, USA).
13. A HiPrep 26/60 Sephacryl S-100 HR column (Amersham Biosciences, Piscataway, NJ, USA).
14. Gel filtration buffer: 25 mM sodium phosphate (pH 7.5), 100 mM NaCl, 10% glycerol. Prepare by mixing 7.1 g sodium phosphate dibasic, 11.69 g NaCl and 200 ml glycerol with H_2O to a volume of 1980 ml. Adjust the pH to 7.5 using concentrated hydrochloric acid. Adjust the volume to 2 l with H_2O and check the pH. Adjust if necessary. Filter through a 0.22 µm polyethersulfone membrane (Corning Incorporated, Corning, NY, USA, or the equivalent) and store at 4°C.
15. 0.2 µm syringe filter (Gelman, Acrodisc Supor membrane, Pall Corporation, Ann Arbor, MI, USA).
16. A Dewar flask filled with liquid nitrogen.

19.3. Methods

19.3.1. Overproduction of Soluble His_6-TEV(S219V)-Arg5 Protease in E. coli

The induction of pRK793 with IPTG produces an MBP fusion protein (*see* **Fig. 19.1**) that self-cleaves *in vivo* to generate a soluble His_6-TEV(S219V)-Arg_5 protease. Virtually all the protease remains soluble after intracellular processing if the temperature is reduced from 37 to 30°C after the addition of IPTG.

1. Inoculate 50–150 ml of LB broth containing 100 µg/ml ampicillin and 30 µg/ml chlorampenicol in a 500 ml baffle-bottom shake flask from a glycerol stock of pRK793 transformed *E. coli* BL21(DE3) CodonPlus-RIL cells. Place in an incubator and shake overnight at 250 rpm and 37°C.
2. Add 25 ml of the saturated overnight culture to each 1 l of fresh LB broth containing 100 µg/ml ampicillin, 30 µg/ml chloramphenicol and 0.2% glucose in a 4 l baffle-bottom shake flask. To ensure that there will be an adequate yield of pure protein at the end of the process, we ordinarily grow 4–6 l of cells at a time.
3. Shake the flasks at 250 rpm and 37°C until the cells reach mid-log phase ($OD_{600\,nm}$ ~0.5); approximately 2 h.

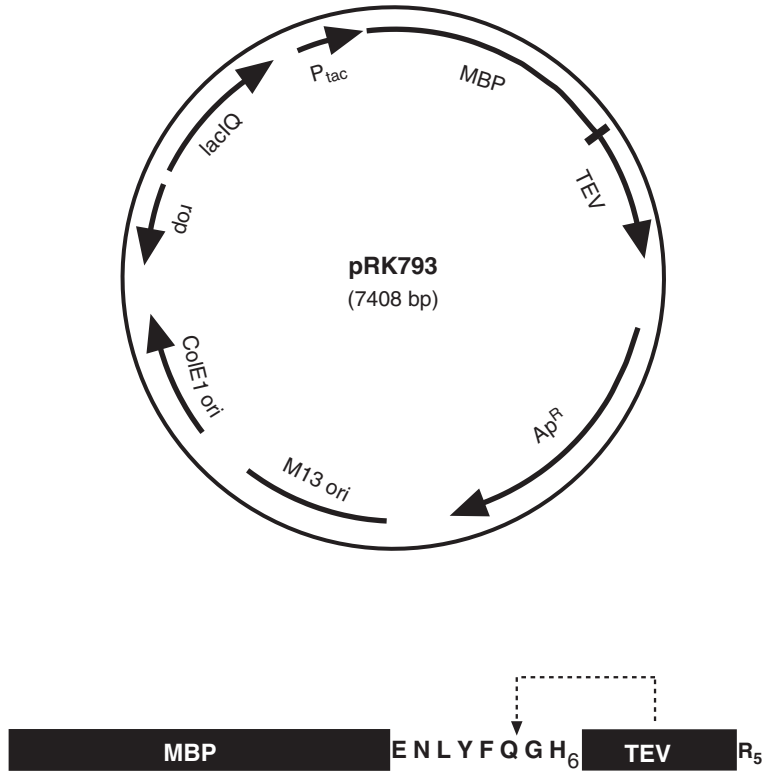

Fig. 19.1. Schematic representation (not to scale) of the TEV protease expression vector pRK793 and its fusion protein product. Further information about this plasmid can be found at http://mcl1.ncifcrf.gov/waugh_tech.html.

4. Shift the temperature to 30°C and induce the culture(s) with IPTG at a final concentration of 1 mM (5 ml of 200 mM IPTG stock solution per liter of culture). Continue shaking at 250 rpm for 4–6 h. Place cultures at 4°C.
5. Recover the cells by centrifugation at 5,000×g for 10 min at 4°C, and store at –80°C. A 6 l preparation yields 30–40 g of cell paste.

19.3.2. Protein Purification

His_6-TEV(S219V)-Arg_5 protease can be purified to homogeneity in two steps: immobilized metal affinity chromatography (IMAC) using Ni-NTA resin followed by size exclusion chromatography. An example of a purification monitored by SDS–PAGE is shown in **Fig. 19.2** (*see* **Note 3**):

1. All procedures are performed at 4–8°C. Thaw the cell paste from 6 l of culture on ice and suspend in ice-cold cell lysis/IMAC equilibration buffer (10 ml/g cell paste).
2. Lyse the cell suspension (see Note 2) and measure the volume using a graduated cylinder. Add polyethylenimine to a final

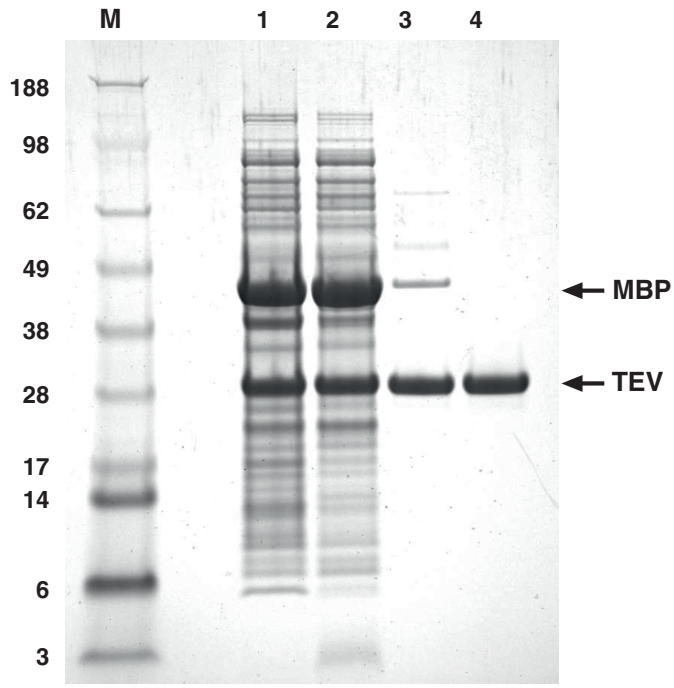

Fig. 19.2. Purification of His_6-TEV(S219V)-Arg_5 protease monitored by SDS–PAGE (NuPage 4–12% gradient MES gel). M: molecular weight standards (kDa). *Lane 1*: Total intracellular protein after induction. *Lane 2*: Soluble cell extract. *Lane 3:* Pooled peak fractions after IMAC. *Lane 4*: Pooled peak fractions after gel filtration and concentration.

concentration of 0.1% (a 1:50 dilution of the 5% stock solution at pH 8) and mix gently by inversion. Immediately centrifuge at 15,000×g for 30 min and filter (*see* **Note 2**).

3. Apply the supernatant to a 50 ml Ni-NTA superflow column equilibrated in cell lysis/IMAC equilibration buffer (*see* **Note 4**). Wash the column with equilibration buffer until a stable baseline is reached (approximately 7 column volumes) and then elute the bound His_6-TEV(S219V)-Arg_5 with a linear gradient to 100% elution buffer over ten column volumes.
4. Pool the peak fractions containing the protease and measure the volume. Add EDTA to a final concentration of 2 mM (a 1:250 dilution of the 0.5 M EDTA, pH 8 stock solution) and mix well. Add DTT to a final concentration of 5 mM (a 1:200 dilution of the 1M DTT stock solution) and mix well.
5. Concentrate the sample approximately tenfold using an Amicon stirred ultrafiltration cell fitted with a YM10 membrane. Remove the precipitation by centrifugation at 5,000×*g* for 10 min. Estimate the concentration of the partially pure protein solution spectrophotometrically at 280 nm

using a molar extinction coefficient of 32,290 M^{-1} cm^{-1}. The desired concentration is between 5 and 10 mg/ml.

6. Apply 5 ml of the concentrated sample onto a HiPrep 26/60 Sephacryl S-100 HR column equilibrated with gel filtration buffer. The volume of sample loaded should be no more than 2% of the column volume and contain no more than 50 mg of protein.
7. Pool the peak fractions from the gel filtration column(s) of pure His_6-TEV(S219V)-Arg_5 protease and concentrate to 1–5 mg/ml (*see* **Step 5** in **Section 19.3.2**). Filter through a 0.2 µm syringe filter (Gelman, Acrodisc Supor membrane, Pall Corporation, Ann Arbor, MI, USA), aliquot and flash freeze with liquid nitrogen. Store at –80°C.

19.3.3. Cleaving a Fusion Protein Substrate with TEV Protease

The standard reaction buffer for TEV protease is 50 mM Tris–HCl (pH 8.0), 0.5 mM EDTA and 1 mM DTT, but the enzyme has a relatively flat activity profile at pH values between 4 and 9 and will tolerate a range of buffers, including phosphate, MES and acetate. TEV protease activity is not adversely affected by the addition of glycerol or sorbitol up to at least 40% (w/v). The enzyme is also compatible with some detergents *(16)*. TEV protease activity is not inhibited by PMSF and AEBSF (1 mM), TLCK (1 mM), Bestatin (1 mg/ml), pepstatin A (1 mM), EDTA (1 mM), E-64 (3 mg/ml), or "complete" protease inhibitor cocktail (Roche). However, zinc will inhibit the activity of the enzyme at concentrations of 5 mM or greater and reagents that react with cysteine (e.g., iodoacetamide) are potent inhibitors of TEV protease.

The duration of the cleavage reaction is typically overnight. A good rule of thumb is to use 1 OD_{280} of TEV protease per 100 OD_{280} of fusion protein for an overnight digest. TEV protease is maximally active at 34°C *(17)*, but we recommend performing the digest at 4°C. The results of a typical TEV protease digest of a fusion protein substrate (MBP-NusG) are shown in **Fig. 19.3.**

Some fusion proteins are intrinsically poor substrates for TEV protease. This may be due to steric occlusion when the protease cleavage site is too close to ordered structure in the passenger protein, or when the fusion protein exists in the form of soluble aggregates. Sometimes this problem can be mitigated by using a large amount of TEV protease (we have occasionally used up to 1 OD_{280} of TEV protease per 5 OD_{280} of fusion protein) and/or performing the reaction at higher temperature (e.g., room temperature). Failing that, the addition of extra residues between the TEV protease cleavage site and the N-terminus of the target protein is advised. We have used polyglycine, polyhistidine and a FLAG-tag epitope with good results.

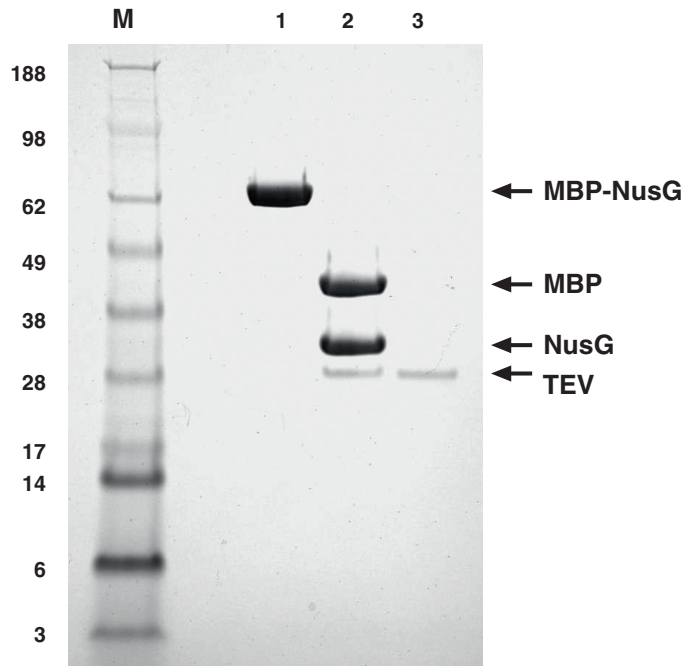

Fig. 19.3. Digestion of a fusion protein substrate by His_6-TEV(S219V)-Arg_5 protease. 22 µg of the substrate, a fusion between *E. coli* maltose-binding protein (MBP) and *Aquifex aeolicus* NusG with a canonical TEV protease recognition site (ENLYFQG) in the linker region *(10)* was incubated for 1 h at room temperature in 50 µl of standard reaction buffer (*see* **Section 19.3.3**) in the absence (*Lane 1*) or presence (*Lane 2*) of 1.3 µg His_6-TEV(S219V)-Arg_5 protease. The reaction products were separated by SDS–PAGE (NuPage 4–12% MES gradient gel) and visualized by staining with Coomassie brilliant blue. *Lane 3* contains an equivalent amount of pure His_6-TEV(S219V)-Arg_5 protease. M: molecular weight standards (kDa).

19.4. Notes

1. *E. coli* BL21(DE3) CodonPlus-RIL cells containing pRK793 can be obtained for a nominal shipping and handling fee from the non-profit distributor of biological reagents AddGene, Inc., Cambridge, MA, USA (http://www.addgene.org) or from the American Type Culture Collection (ATCC catalog number MB-145). The pRIL plasmid is a derivative of the p15A replicon that carries the *E. coli argU*, *ileY* and *leuW* genes, which encode the cognate tRNAs for AGG/AGA, AUA and CUA codons, respectively. pRIL is selected for by resistance to chloramphenicol. Due to the presence of several AGG and AGA codons in the TEV protease coding sequence, the presence of pRIL dramatically increases the yield of TEV protease.
2. We routinely break cells using an APV-1000 homogenizer (Invensys, Roholmsvej, Denmark) at 10–11,000 psi for 2–3

rounds. Other homogenization techniques such as French press, sonication, or manual shearing should yield comparable results. Centrifugation of the disrupted cell suspension for at least 30 min at 30,000×*g* is recommended. Filtration through a 0.45 µm polyethersulfone (or cellulose acetate) membrane is helpful to remove residual particulates and fines prior to chromatography.

3. We find it convenient to use precast gels for SDS–PAGE gels (e.g., 1.0 mm × 10 well, 4–12% NuPage gradient), running buffer and electrophoresis supplies from Invitrogen (Carlsbad, CA, USA).
4. We use an ÄKTA Explorer chromatography system (Amersham Biosciences, Piscataway, NJ, USA) and a Ni-NTA Superflow column (Qiagen, Valencia, CA, USA). A properly poured 50 ml Ni-NTA Superflow column (in an Amersham Biosciences XK26/20 column) can be run at 4–6 ml/min (backpressure less than 0.4 MPa) and will bind up to 400 mg of His_6-TEV(S219V)-Arg5 protease. If a chromatography system is not available, the IMAC can be performed using a peristaltic pump or manually by gravity. If the latter is used, Ni-NTA agarose should be substituted for Superflow and the elution performed with step increases of imidazole in 25 mM increments. Binding and elution profiles can be monitored spectrophotometrically at 280 nm and by SDS–PAGE. Care must be taken to properly zero the spectrophotometer because imidazole has significant absorption in the UV range.

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