TEV Protease FAQ

What is TEV protease and why would I use it?

TEV protease is the common name for the 27 kDa catalytic domain of the Nuclear Inclusion a (NIa) protein encoded by the tobacco etch virus (TEV). Because its sequence specificity is far more stringent than that of factor Xa, thrombin, or enterokinase, TEV protease is a very useful reagent for cleaving fusion proteins. It is also relatively easy to overproduce and purify large quantities of the enzyme.

What is the cleavage site for TEV protease?

TEV protease recognizes a linear epitope of the general form $E-X_{aa}-X_{aa}-Y-X_{aa}-Q-(G/S)$, with cleavage occurring between Q and G or Q and S. The most commonly used sequence is ENLYFQG. Early work by the Dougherty lab suggested that the protease will readily tolerate many different amino acid side chains in the P5, P4 and P2 positions (Xaa) of its substrates. Indeed, the natural cleavage sites in the TEV polyprotein exhibit some variability in these positions. However, we have found that even relatively conservative substitutions in the P4 (Leu to Phe) and P2 (Phe to Tyr) positions reduce processing efficiency *in vitro* by approximately 2 orders of magnitude [Tözsér *et al.*, manuscipt in preparation]. The crystal structure of TEV protease in complex with an oligopeptide substrate [Phan *et al.*, 2002] revealed that the only side-chain in the canonical recognition site (ENLYFQS) that does not make intimate contact with the enzyme is P5 Asn. A systematic study demonstrated that many different amino acids can be accommodated in the P1' position of a model fusion protein substrate with relatively little impact on processing efficiency [Kapust *et al.*, 2002a], most likely because the S1' subsite in the enzyme is a shallow groove on its surface rather than a true pocket. However, the most efficient substrate was ENLYFQS.

What is known about the enzymatic mechanism of TEV protease?

The structure of TEV protease is similar to those of serine proteases like chymotrypsin [Phan *et al.*, 2002]. Like the serine proteases, TEV protease utilizes a "catalytic triad" of residues to catalyze peptide hydrolysis. However, in TEV protease the serine nucleophile of the conventional Ser-Asp-His triad is a cysteine instead. This probably explains why TEV protease is resistant to many commonly used protease inhibitors.

What protease inhibitors are known not to affect TEV?

PMSF and AEBSF (1mM), TLCK (1mM), Bestatin (1mg/ml), pepstatin A (1mM), EDTA (1mM), and E-64(3mg/ml), "complete" protease inhibitor cocktail (Roche). Zinc will inhibit the activity of the enzyme at concentrations of 5 mM or greater. Reagents that react with cysteine (*e.g.*, iodoacetamide) are potent inhibitors of TEV protease.

What forms of TEV protease are available?

The commercial version of TEV protease (Invitrogen) consists of the 27 kDa catalytic domain with an N-terminal polyhistidine tag. A serious drawback of this form of TEV protease, apart from its cost, is that it readily cleaves itself at a specific site to yield a truncated enzyme with greatly diminished activity. A more stable mutant (S219N) of TEV protease, also with an N-terminal polyhistidine tag, was described by the Doudna laboratory [Lucast *et al.*, 2001]. This form of TEV protease was refolded from inclusion bodies. We have constructed several TEV protease mutants that are highly resistant to autolysis [Kapust et al., 2001]. The S219V mutant is impervious to autoinactivation under normal reaction conditions and is also a slightly more efficient catalyst than the wild-type enzyme. We estimate that it is at least ten-fold more stable than the S219N mutant. The S219P mutant does not undergo autolysis under any conditions, but is two-fold less active than the wild-type enzyme. Two different forms of the S219V mutant are available from our lab: one with an N-terminal polyhistidine tag and a C-terminal polyarginine tag, and another with an N-terminal maltose-binding protein tag and a C-terminal polyarginine tag.   Both forms can be produced in a high yield in *E. coli* as soluble proteins that do not require refolding [Kapust *et al.,* 1999, 2001, 2002b].

What are typical reaction conditions?

The "standard" reaction buffer for TEV protease is 50 mM Tris-HCl (pH 8.0), 0.5 mM EDTA and 1mM DTT. The duration of the cleavage reaction is typically overnight, although lots of cleavage will happen in the first few hours and prolonged incubation times may not lead to proportional increases in cleavage. TEV protease is maximally active at 34 °C, but we recommend performing the digest at room temperature (20 °C) or 4 °C. TEV protease is only three-fold less active at 4 °C than at 20 °C (Nallamsetty *et al*., manuscript in preparation).

How much TEV protease should I use?

A good rule of thumb is 1 OD_{280} of TEV protease per 100 OD_{280} of substrate for an overnight digest. Perform a small-scale reaction first, if possible, to gauge the efficiency of processing. In especially difficult cases, we have used as much as 1 OD_{280} of TEV protease per 5 OD_{280} of substrate. This is feasible because TEV protease is not a promiscuous enzyme.

Can I do an on-column cleavage?

On-column cleavage is possible but comparatively inefficient. TEV protease is not especially stable and has a tendency to bind nonspecifically to column materials. The addition of "stabilizers" like sorbitol, glycerol, or salt may be helpful.

Why won't TEV protease cleave my fusion protein?

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 target 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. 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 in this position with good results.

How sensitive is TEV protease to other additives and reaction parameters?

TEV protease has a relatively flat activity profile at pH values between 4 and 9. It is 50% as active in 0.5 M NaCl as it is in the absence of salt (our unpublished observations). TEV protease is maximally active at 34 °C, but we recommend performing digests at room temperature (20 °C) or 4 °C. The activity of TEV protease is approximately 3-fold greater at 20 °C than at 4 °C (our unpublished observations). It will tolerate a range of buffers, including phosphate, MES, and acetate. TEV protease is not adversely affected by the addition of glycerol or sorbitol (up to at least 40% w/v). TEV protease is sensitive to some detergents [Mohanty *et al.,* 2003].

Why should I consider cleaving my fusion protein with TEV protease *in vivo*?

There are at least two good reasons to do this. First, it will allow you to determine, to a first approximation, how efficiently your fusion protein will be cleaved *in vitro*. Armed with this information, you can make an educated guess about how much protease to use. Second, you can get an early indication of how your protein will behave after it is released from its fusion partner. Some large fusion partners (*e.g.*, MBP) are remarkably effective solubilizing agents. However, in some cases the passenger proteins are only soluble (protected from aggregating) as long as they are fused to MBP (see MBP fusion protein FAQ). If the passenger protein is insoluble after the fusion protein is cleaved *in vivo*, then it is likely that this will also be the case *in vitro*. It is also worth noting that if the construct is designed so that the target protein retains an affinity tag (*e.g.*, polyhistidine) after the fusion protein is cleaved by TEV protease, then in the end it may be less work to cleave it *in vivo* and work up the target protein using only the secondary affinity tag. Methods for intracellular processing of fusion proteins by TEV protease have been described [Kapust & Waugh, 2000; Fox & Waugh, 2003].

<u>What about disulfide bonds?</u>

If the target protein is expected to contain disulfide bonds, DTT should not be used in the reaction. Under these conditions, DTT can be replaced with a redox buffer like 3 mM glutathione/0.3 mM oxidized glutathione, which should maintain the disulfide bonds while providing enough reducing power for TEV protease to work.

<u>What about zinc fingers?</u>

Both EDTA and DTT are strong metal chelators, and consequently they might be expected to strip the zinc ions from weaker zinc finger motifs. We recommend that DTT be replaced by a monothiol such as glutathione or beta-mercaptoethanol when working with zinc fingers. EDTA should be replaced by a weaker metal chelator such as citrate. Ming Zhou from the Morrison laboratory at NCI-Frederick found that the following buffer supports TEV protease activity and is expected to be very kind to zinc fingers:

> 20 mM Tris-HCl, pH 7.4
> 10 μM $ZnCl_2$
> 200 mM NaCl
> 5 mM citrate
> 5 mM 2-mercaptoethanol

<u>How can I remove TEV protease after digesting a fusion protein substrate?</u>

When working with an affinity-tagged form of TEV protease, an obvious option is to absorb it to the appropriate affinity resin. Don't forget that DTT and EDTA are incompatible with IMAC and therefore must be removed first. Because of the C-terminal polyarginine tag, both forms of the S219V mutant TEV protease that we use will bind very tightly to a cation exchange resin, even at pH 8-9. Under these conditions, the vast majority of proteins will fail to adhere to the resin. Gel filtration may also work, depending on the size of the target protein and the form of TEV protease used.

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