

The variable detergent sensitivity of proteases that are utilized for recombinant protein affinity tag removal

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ABSTRACT

Recombinant proteins typically include one or more affinity tags to facilitate purification and/or detection. Expression constructs with affinity tags often include an engineered protease site for tag removal. Like other enzymes, the activities of proteases can be affected by buffer conditions. The buffers used for integral membrane proteins contain detergents, which are required to maintain protein solubility. We examined the detergent sensitivity of six commonly-used proteases (enterokinase, factor Xa, human rhinovirus 3C protease, SUMOstar, tobacco etch virus protease, and thrombin) by use of a panel of 94 individual detergents. Thrombin activity was insensitive to the entire panel of detergents, thus suggesting it as the optimal choice for use with membrane proteins. Enterokinase and factor Xa were only affected by a small number of detergents, making them good choices as well.

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Introduction

Modern recombinant protein expression constructs include one or more affinity tags to aid in purification and/or detection. After serving its requisite function(s), the tag is often removed so as not to (potentially) interfere with “downstream” protein applications such as functional or structural studies. Three-dimensional crystallization, for structure determination by X-ray crystallography, is often deleteriously affected by inclusion of the disordered or flexible affinity tag. An engineered site for a specific protease in the linker region between tag(s) and native protein is thus included to facilitate tag removal. Common proteases include enterokinase [1], factor Xa [2], human rhinovirus 3C protease (HRV 3C)² [3], SUMO protease [4], tobacco etch virus (TEV) protease [5], and thrombin [6,7]. Table 1 lists the canonical recognition sequences, and specific cut-sites, for each of these proteases. For constructs containing an N-terminal tag with a protease site in the linker, enterokinase, factor Xa, and SUMOstar will return the original (parent) protein, while HRV 3C, thrombin, and TEV leave several residues of the protease site. TEV is the most widely-used of these proteases [8–11]. In addition to its high specificity, TEV

maintains activity in a wide range of buffer and solution conditions, and is readily capable of being produced in-house.

Several other considerations can influence the choice of protease for removal of affinity tags. Protease specificity can vary widely. Digestive and coagulation proteases can (and do) cleave proteins at sites other than the engineered “cut-site”; examples of this include non-specific proteolysis of recombinant proteins by enterokinase [12], thrombin [13] and factor Xa [13]. To quote, “it is necessary to characterize the protein of interest after cleavage from the affinity label to assure that there are no changes in the covalent structure of the protein of interest [13]”. Typically, this characterization method would be mass spectrometry, and reliable methods of sample preparation have been developed for integral membrane proteins [14]. In contrast, viral proteases (e.g. HRV 3C and TEV) are very highly specific [15,16]. However, viral proteases typically possess turnover rates that are very much lower, as much as 10⁴ lower, than those of non-viral proteases [17]. The much lower activity of viral proteases is reflected, empirically, in the observation that those labs which utilize it for “large-scale” protein production (for X-ray crystallography or NMR spectroscopy) commonly make their own HRV 3C and/or TEV for use. Therefore, the selection of non-viral vs. viral proteases, for removal of affinity tags from recombinant fusion proteins, is, essentially, a trade-off between specificity and activity.

Maintaining the solubility of integral membrane proteins in aqueous solution requires the presence of detergents or other surfactants [18]. These detergents, present at concentrations above the critical micelle concentration (CMC), form a protein-detergent complex (PDC) with the membrane protein [19]. Detergents can have inhibitory effects upon proteases; in one example, we

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² Abbreviations used: CMC, critical micelle concentration; DTT, dithiothreitol; EDTA, ethylenediamine-tetraacetic acid; HRV 3C, human rhinovirus 3C protease; PAGE, polyacrylamide gel electrophoresis; PDC, protein-detergent complex; SUMO, small ubiquitin-like modifier; TEV, tobacco etch virus protease.

Table 1
Proteases used in this study.

Protease	Cleavage site
Enterokinase	Asp-Asp-Asp-Lys ∇
Factor Xa	Ile-Glu/Asp-Gly-Arg ∇
HRV 3C	Leu-Glu-Val-Leu-Phe-Gln ∇ Gly-Pro
SUMOstar	Recognizes tertiary structure of SUMOstar tag (10 kDa)
TEV	Glu-Asn-Leu-Tyr-Phe-Gln ∇ Gly/Ser
Thrombin	Leu-Val-Pro-Arg ∇ Gly-Ser

The amino acid recognition site for each protease is provided with the site of cleavage indicated by the ∇ . All proteases recognize short, linear sequences while SUMOstar additionally recognizes the tertiary structure of the SUMOstar tag.

previously demonstrated that several detergents inhibit TEV [20]. The inability of TEV to efficiently remove an affinity tag in a particular detergent is troublesome and unfortunately precludes the universal use of TEV for membrane proteins. Many detergents and detergent mixtures are, in principle, possible candidates for use with membrane proteins. Also, as mentioned, multiple proteases besides TEV are commonly used. In practice, when a protease does not remove the affinity tag of a membrane protein, two possibilities (that are not mutually exclusive) for this failure exist. The tag could be sterically inaccessible to the protease because of the protein, the detergent, or both. Or, the protease could be inhibited by the detergent. In order to eliminate this situation of “one equation with two unknowns”, we characterized the sensitivities of a set of proteases (enterokinase, factor Xa, HRV 3C, SUMOstar, TEV, and thrombin) to a large number (94) of individual detergents. This detergent panel was recently compiled in conjunction with our recent development of a high-throughput assay for screening the stability and size of a PDC in multiple detergents [21].

Materials and methods

Materials

Enterokinase, factor Xa, HRV 3C, and thrombin along with their respective cleavage control proteins were purchased from EMD

Biosciences; SUMOstar and its cleavage control protein were obtained from LifeSensors, Inc. We made TEV “in-house” using published methods [22]; the cleavage control protein is a protein domain on which we work [23], and its affinity tag is quantitatively removed by TEV [24]. Detergents were from Anatrace, Avanti Polar Lipids, EMD Biosciences, or Bachem. Electrophoresis and blotting were performed with E-PAGE 48-well 8% gels and iBLOT nitrocellulose transfer stacks (Invitrogen), and visualized with colloidal gold total protein stain (Bio-Rad).

Protease digestion

Enterokinase (1:50 dilution, 4 h digest, 1 μ g control protein/well); factor Xa (1:50 dilution, 4 h digest, 2 μ g control protein/well); HRV 3C (1:50 dilution, 4 h digest, 1 μ g control protein/well); SUMOstar (1:50 dilution, 4 h digest, 2 μ g control protein/well); TEV (36 ng/ μ l, overnight digest, 5 μ g control protein/well); thrombin (1:35 dilution, 4 h digest, 1 μ g control protein/well). The reaction and dilution buffers were made from the concentrated commercial stocks accompanying the proteases except for TEV where the buffer (100 mM Tris pH 8, 1 mM EDTA, 2 mM DDT) was prepared. Two samples were made in 96 well PCR plates based on the above conditions. Plate 1 contained control protein and the detergent while plate 2 consisted of the control protein, detergent, and 1 μ l of the diluted protease. The final volume of each well was 15 μ l. Plates were gently shaken at 25 $^{\circ}$ C/300 rpm in an Eppendorf Thermomixer. After the digestion was complete, 5 μ l of 4 \times E-PAGE loading dye was added to each plate. Samples were then loaded on a 48-well E-PAGE gel, blotted to a nitrocellulose membrane using the iBLOT apparatus, and visualized with colloidal gold stain.

Results and discussion

In order to assess the activity of commonly used proteases in our detergent panel, we digested soluble proteins containing the appropriate protease cleavage site. The experimental design presented here is similar to our previous study of the detergent sensitivity of TEV [20]. We note that a report from another laboratory

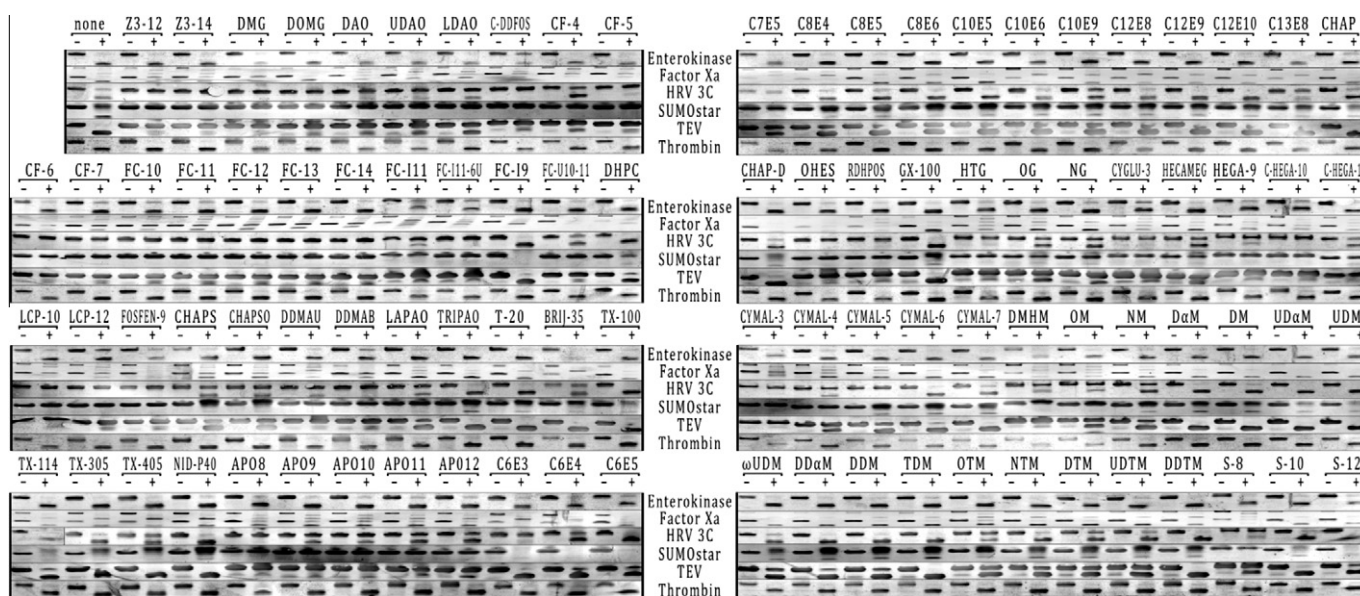


Fig. 1. Gel lanes for each protease experiment are shown above labeled “-” for no protease and “+” for protease present. The abbreviations for the detergents are given in Table 2. The rows were cut out from scanned images of the 48-well blots and their contrast was adjusted automatically within Adobe Photoshop CS2. All control protease control proteins showed a simple gel shift after digestion with the exception of the factor Xa control protein which formed SDS-resistant oligomers. These oligomers did not prevent analysis of the results. The amount of digestion was estimated from the amount of digested protein formed in the protease “+” lane compared to the protease “-” lane and assigned a value of “+++”, “++”, “+”, or “-”. The image for TX-114 for HRV 3C was repeated from another blot due to a bubble in the original transfer.

Table 2
Summary of detergent sensitivity of proteases.

Name	[Det] mM	Abbrev.	Enterokinase	Factor Xa	HRV 3C	SUMOstar	TEV	Thrombin
ZWITTERGENT® 3-12	8.4 (2.8)	Z3-12						
ZWITTERGENT® 3-14	10 (0.2)	Z3-14						
n-Decyl-N,N-dimethylglycine	38 (19)	DMG						
n-Dodecyl-N,N-dimethylglycine	4.5 (1.5)	DOMG						
n-Decyl-N,N-dimethylamine-N-oxide	21 (10.5)	DAO						
n-Undecyl-N,N-dimethylamine-N-oxide	9.6 (3.2)	UDAO						
n-Dodecyl-N,N-dimethylamine-N-oxide	3 (1)	LDAO						
C-DODECAFOS™	44 (22)	C-DDFOS						
CYCLOFOS™-4	28 (14)	CF-4						
CYCLOFOS™-5	13.5 (4.5)	CF-5						
CYCLOFOS™-6	8.04 (2.68)	CF-6						
CYCLOFOS™-7	6.2 (0.62)	CF-7						
FOS-CHOLINE®-10	22 (11)	FC-10						
FOS-CHOLINE®-11	5.55 (1.85)	FC-11						
FOS-CHOLINE®-12	4.5 (1.5)	FC-12						
FOS-CHOLINE®-13	7.5 (0.75)	FC-13						
FOS-CHOLINE®-14	6 (0.12)	FC-14						
FOS-CHOLINE®-ISO-11	53.2 (26.6)	FC-I11						
FOS-CHOLINE®-ISO-11-6U	51.6 (25.8)	FC-I11-6U						
FOS-CHOLINE®-ISO-9	64 (32)	FC-I9						
FOS-CHOLINE®-UNSAT-11-10	15.5 (6.2)	FC-U10-11						
1,2-Diheptanoyl-sn-glycero-3-phosphocholine	4.2 (1.4)	DHPC						
LysoPC-10	20 (8)	LPC-10						
LysoPC-12	7 (0.7)	LPC-12						
FOSFEN™-9	4.05 (1.35)	FOSFEN-9						
CHAPS	20 (8)	CHAPS						
CHAPSO	20 (8)	CHAPSO						
n-Dodecyl-N,N-(dimethylammonio)undecanoate	6.5 (0.13)	DDMAU						
n-Dodecyl-N,N-(dimethylammonio)butyrate	12.9 (4.3)	DDMAB						
LAPAO	4.8 (1.6)	LAPAO						
TRIPAO	13.5 (4.5)	TRIPAO						
TWEEN® 20	5.9 (0.059)	T-20						
BRIJ®35	9.1 (0.091)	BRIJ-35						
TRITON® X-100	11.5 (0.23)	TX-100						
TRITON® X-114	10 (0.2)	TX-114						
TRITON® X-305	6.5 (0.65)	TX-305						
TRITON® X-405	8.1 (0.81)	TX-405						
[Octylphenoxy]polyethoxyethanol	15 (0.3)	NID-P40						
Dimethyloctylphosphine oxide	80 (40)	APO8						
Dimethylnonylphosphine oxide	20 (10)	APO9						
Dimethyldecylphosphine oxide	14.0 (4.7)	APO10						
Dimethylundecylphosphine oxide	3.6 (1.2)	APO11						
Dimethyldodecylphosphine oxide	5.7 (0.57)	APO12						
Triethylene glycol monohexyl ether	46 (23)	C6E3						
Tetraethylene glycol monohexyl ether	60 (30)	C6E4						
Pentaethylene glycol monohexyl ether	74 (37)	C6E5						
Pentaethylene glycol monoheptyl ether	42 (21)	C7E5						
Tetraethylene glycol monoethyl ether	20 (8)	C8E4						
Pentaethylene glycol monoethyl ether	17.75 (7.1)	C8E5						
Hexaethylene glycol monoethyl ether	25 (10)	C8E6						
Pentaethylene glycol monodecyl ether	8.1 (0.81)	C10E5						
Hexaethylene glycol monodecyl ether	9 (0.9)	C10E6						
Polyoxyethylene(9)decyl ether	3.9 (1.3)	C10E9						
Octaethylene glycol monododecyl ether	9 (0.09)	C12E8						
Polyoxyethylene(9)dodecyl ether	5 (0.05)	C12E9						
Polyoxyethylene(10)dodecyl ether	10 (0.2)	C12E10						
Polyoxyethylene(8)tridecyl ether	10 (0.1)	C13E8						
Big CHAP	8.7 (2.9)	CHAP						
Big CHAP, deoxy	4.2 (1.4)	CHAP-D						
<i>Octyl-2-hydroxyethyl-sulfoxide</i>	48.4 (24.2)	OHES						
<i>Rac-2,3-dihydroxypropyloctylsulfoxide</i>	48.4 (24.2)	RDHPOS						
Genapol® X-100	7.5 (0.15)	GX-100						
n-Heptyl-β-D-thioglucopyranoside	58 (29)	HTG						
n-Octyl-β-D-glucopyranoside	36 (18)	OG						
n-Nonyl-β-D-glucopyranoside	16.25 (6.5)	NG						
CYGLU®-3	56 (28)	CYGLU-3						
HECAMEG	39 (19.5)	HECAMEG						
Hega®-9	78 (39)	HEGA-9						
C-Hega®-10	70 (35)	C-HEGA-10						
C-Hega®-11	23 (11.5)	C-HEGA-11						
CYMAL®-3	60 (30)	CYMAL-3						
CYMAL®-4	19 (7.6)	CYMAL-4						
CYMAL®-5	7.2 (2.4)	CYMAL-5						
CYMAL®-6	5.6 (0.56)	CYMAL-6						
CYMAL®-7	9.5 (0.19)	CYMAL-7						
2,6-Dimethyl-4-heptyl-β-D-maltoside	55 (27.5)	DMHM						
n-Octyl-β-D-maltopyranoside	39 (19.5)	OM						
n-Nonyl-β-D-maltopyranoside	15 (6)	NM						
n-Decyl-β-D-maltopyranoside	4.8 (1.6)	DαM						
n-Decyl-α-D-maltopyranoside	5.4 (1.8)	DM						
n-Undecyl-α-D-maltopyranoside	5.8 (0.58)	UDαM						
n-Undecyl-β-D-maltopyranoside	5.9 (0.59)	UDM						
ω-Undecylenyl-β-D-maltopyranoside	3.6 (1.2)	ωUDM						
n-Dodecyl-α-D-maltopyranoside	7.5 (0.15)	DDαM						
n-Dodecyl-β-D-maltopyranoside	8.5 (0.17)	DDM						
n-Tridecyl-β-D-maltopyranoside	1.5 (0.03)	TDM						
n-Octyl-β-D-thiomaltopyranoside	21.25 (8.5)	OTM						
n-Nonyl-β-D-thiomaltopyranoside	9.6 (3.2)	NTM						
n-Decyl-β-D-thiomaltopyranoside	9 (0.9)	DTM						
n-Undecyl-β-D-thiomaltopyranoside	10.5 (0.21)	UDTM						
n-Dodecyl-β-D-thiomaltopyranoside	5 (0.05)	DDTM						
Sucrose⁸	48.8 (24.4)	S-8						
Sucrose¹⁰	7.5 (2.5)	S-10						
Sucrose ¹²	15 (0.3)	S-12						



The membrane protein detergent panel is shown above. The values in parenthesis in the [Det] column are the CMC values for each detergent. Detergents in bold were purchased from Avanti Polar Lipids, italics from Bachem, underlined from EMD Biosciences, and all others from Anatrache. The legend shows the relative protease activity in each detergent based on the amount of cleavage product observed on the protein gel.

utilized three different membrane proteins as test proteins [25]. We have chosen to use soluble proteins for several reasons: (1) a test membrane protein would have to be stable in every detergent in the panel to be a reliable test protein, and (2) a protease site on a membrane protein might be occluded by the detergent of the PDC, while a soluble protein should not interact with detergent and is

thus much less likely to have its protease site occluded by detergent. Moreover, in this present study, the use of vendor-supplied positive control proteins obviates the possibility of the protein occluding the cleavage site.

Fig. 1 shows a composite of the protein gels used to evaluate the protease activity in the detergent panel. The relative activities of

each protease were estimated from the amount of cleavage product observed on the protein gels and is summarized in Table 2. The best protease was thrombin which has maximum activity in all of the detergents tested, followed closely by enterokinase and factor Xa, while HRV 3C and SUMOstar were drastically affected by detergent. TEV possessed activity in most detergents, but at low levels in a large percentage of these detergents. Since TEV is typically made as a reagent in-house, more can be added to a cleavage reaction to possibly overcome the inhibitory effect of a particular detergent. The poor performance of SUMOstar was somewhat surprising, since this protease recognizes the tertiary structure of the large SUMOstar tag [4] compared to the short recognition sequences of the other proteases tested. The SUMOstar tag may be partially unfolded in detergent micelle solutions or may possibly insert into the micelle, making it unavailable for binding the SUMOstar protease.

Conclusion

Based upon our data, the activity of thrombin is not significantly affected by any of the 94 detergents of our panel [21]. This panel encompasses, as single detergents in individual solutions, nearly all of the detergents utilized in membrane protein biochemistry, biophysics and structural biology (at present). Therefore, we recommend the design and utilization of a thrombin cleavage site for protein expression constructs; this will provide for the most detergent-invariant affinity tag removal. Moreover, enterokinase and factor Xa were only affected by a small number of detergents, making them good choices as well. Additionally, removal of an N-terminal affinity-binding site by enterokinase or factor Xa produces the wildtype (or parent) construct protein free from any extraneous residues derived from the protease recognition site. This attribute may be (very) advantageous; for example, a crystal contact mediated through the N-terminus could be disrupted by the presence of these extra residues.

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