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Inhibition of tobacco etch virus protease activity by detergents

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Abstract

Affinity tags such as polyhistidine greatly facilitate recombinant protein production. The solubility of integral membrane proteins is maintained by the formation of protein–detergent complexes (PDCs), with detergent present at concentration above its critical micelle concentration (CMC). Removal of the affinity tag necessitates inclusion of an engineered protease cleavage site. A commonly utilized protease for tag removal is tobacco etch virus (TEV) protease. TEV is available in a recombinant form (rTEV) and frequently contains its own polyhistidine affinity tag for removal after use in enzymatic digestion. Proteolytic cleavage of the tagged domain is carried out by incubation of the protein with rTEV protease. We have observed that the efficiency of rTEV digestion decreases significantly in the presence of a variety of detergents utilized in purification, crystallization, and other biochemical studies of integral membrane proteins. This reduction in protease activity is suggestive of detergent-induced inhibition of rTEV. To test this hypothesis, we examined the effects of detergents upon the rTEV proteolytic digestion of a soluble fusion protein, α_1 platelet activating factor acetylhydrolase (PAFAHα_1). Removal of a hexahistidine amino-terminal affinity tag has been characterized in the presence of 16 different detergents at concentrations above their respective CMCs. Our data indicate that half of the detergents tested reduce the activity of rTEV and that these detergents should be avoided or otherwise accounted for during rTEV digestion of recombinant integral membrane proteins.

Biochemical and structural characterization of integral membrane proteins require multimilligram quantities of purified protein. Recombinant DNA technology has facilitated the design and development of various vector DNA based systems for high-level expression of foreign genes in different hosts. For the purification of recombinant proteins, various classical separation procedures are employed and are based on the physical-chemical properties of the protein, such as charge, size, and hydrophobicity. These procedures are often time consuming and laborious; therefore, simple and rapid alternatives for purification have been developed. The production of recombinant proteins containing polyhistidine tags has become a frequently used procedure for rapid purification of recombinant proteins [1,2]. This affinity tagging is readily performed by fusion of a cDNA that codes for polyhistidine (typically six to ten histidines) to the gene of interest in the expression vector, which yields a protein that can be detected and/or purified via immobilized metal affinity chromatography (IMAC) or anti-histidine antibodies. Purification of many bacterially expressed integral membrane proteins has been reported (for example, glucose transporter [3], diacylglycerol kinase [4], outer membrane iron siderophore transporter FhuA [5], nontypeable *Haemophilus influenzae* p5 outer membrane protein [6], CLC chloride channel homolog YadQ [7], and acyl–acyl carrier protein synthase [8]). Removal of the affinity tag from the protein of interest is frequently sought and is accomplished by inserting a site-specific protease cleavage site between the tag and the protein. The tag is then separated from the fusion protein by proteolysis after affinity chromatography. Many different proteases are used for cleavage including Factor Xa [9], thrombin [10,11], enterokinase [12], preScission [13], or tobacco etch virus

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Table 1
Effect of detergent on rTEV protease digestion

Detergent	Detergent concentration during rTEV digestion (mM)	Detergent CMC (mM)[a]	Detergent chain length	Detergent type[b]	Digestion[c]
No detergent	—	—	—	—	C
APO-10	9	4.57[d]	10	N	I
C_8E_4	20	7.2[e]	8	N	C
DDMAB	9	4.3[d]	12	Z	I
DHPC	4	1.4[f]	7	Z	C
DM	5	1.8[e]	10	N	C
DODMG	5	1.5[e]	12	Z	I
DS	9	2.5[d]	10	N	C
FC-12	5	1.5[e]	12	Z	I
FOS-MEA-10	10	5.25[e]	10	Z	C
HECAMEG	35	19.5[e]	7	N	I
HEGA-10	20	7.0[e]	10	N	C
LDAO	5	1.4[e]	12	Z	I
LYSOPC-12	3	0.9[f]	12	Z	C
NTM	6.5	3.2[e]	9	N	C
OG	35	19.0[e]	8	N	I
ZW 3–12	6.5	3.0[d]	12	Z	I

[a] Detergent CMC values were obtained from the catalogs of Calbiochem, CA, Anatrace, OH, Avanti Polar Lipids, AL.
[b] N—Neutral, Z—zwitterionic.
[c] C—Complete, I—incomplete.
[d] Detergent CMC values were obtained from the catalogs of Calbiochem, CA.
[e] Detergent CMC values were obtained from the catalogs of Anatrace, OH.
[f] Detergent CMC values were obtained from the catalogs of Avanti Polar Lipids, AL.

(TEV)[1] protease [14]. TEV protease is among the most widely used due to its high specificity and its activity over a broad temperature range [15]. It has been cloned as a polyhistidine tagged fusion protein [14] and has been engineered to increase its expression level in *Escherichia coli* [16].

The focus of our laboratory is structural biology (X-ray crystallography) of integral membrane proteins. The proteins are typically purified as affinity tagged fusion proteins in buffers that contain various detergents at concentrations above their respective critical micelle concentrations (CMCs). A solution of purified membrane protein contains, in equilibrium, protein–detergent complexes (PDCs), free detergent micelles, and detergent monomers [17]. We have frequently observed incomplete tag removal from recombinant membrane proteins after treatment with rTEV protease. Variation of digestion time, digestion temperature, and amount of protease often had relatively little effect upon efficiency or yield. This effect suggests the possibility of inhibition of rTEV protease activity due to detergents at concentrations above their CMCs. To address this possibility, we examined the effects of detergents upon the rTEV protease cleavage of a polyhistidine affinity tag from the recombinant soluble protein α_1 platelet activating factor acetylhydrolase (PAFAHα_1) [18]. This protein has an amino hexahistidine tag with an rTEV protease cleavage site and is digested quantitatively by rTEV. The tag removal has been characterized in the presence of 16 different detergents. Our results indicate that certain detergents, namely; APO-10, DDMAB, DODMG, FC-12, HECAMEG, LDAO, OG, and ZW 3–12 (Table 1) can reduce rTEV digestion to varying degrees. These detergents may be avoided when using rTEV digestion as a method of tag removal from recombinant membrane proteins.

Materials and methods

Materials

The plasmid pHis-PAFAHα_1 encoding α_1-platelet activating factor acetylhydrolase was provided by

[1] *Abbreviations used:* PDC, protein–detergent complex; CMC, critical micelle concentration; TEV, tobacco etch virus; rTEV, recombinant tobacco etch virus; PAFAHα_1, α_1 platelet activating factor acetylhydrolase; IMAC, immobilized metal affinity chromatography; EDTA, ethylenediaminetetraacetic acid disodium salt dihydrate; LB, Luria–Bertani; IPTG, isopropyl-β-D-thiogalactopyranoside; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; LDS, lithium dodecyl sulfate; APO-10, dimethyldecylphosphine oxide; C_8E_4, tetraethyleneglycolmonooctylether; DDMAB, *n*-dodecyl-*N*,*N*-(dimethylammonio)butyrate; DHPC, 1,2-diheptanoyl-sn-glycero-3-phosphocholine; DM, *n*-decyl-β -D-maltopyranoside; DODMG, *n*-dodecyl-*N*,*N*-dimethylglycine; DS, *n*-decanoyl-β-D-fructofuranosyl-α-D-glucopyranoside; FC-12, *n*-dodecylphosphocholine; FOS-MEA-10, decyl-phospho-*n*-methylethanolamine; HECAMEG, methyl-6-*O*-(*N*-heptylcarbamoyl)-α-D-glucopyranoside; HEGA-10, decanoyl-*N*-hydroxyethylglucamide; LDAO, *n*-dodecyl-*N*,*N*-dimethylamine-*N*-oxide; LYSOPC-12, 1-lauroyl-sn-glycero-3-phosphocholine; NTM, *n*-nonyl-β-D-thiomaltopyranoside; OG, *n*-octyl-β-D-glucopyranoside; ZW 3–12, 3-(dodecyldimethylammonio)-1-sulfonate.

Dr. Zygmunt Derewenda (University of Virginia). The *E. coli* strain BL21(DE3)pLysS was used for protein expression. Isopropyl-β-D-thiogalactopyranoside (IPTG) was obtained from Research Products International, IL. Detergents were purchased from Anatrace, OH; Avanti Polar Lipids, AL; or Calbiochem, CA. TransformAid transformation kit was from MBI Fermentas, MD. Ni-NTA Superflow metal affinity resin was from Qiagen, CA. Recombinant tobacco etch virus (rTEV) protease was from Life Technologies, MD. Novex NuPAGE (10%) Bis–Tris gels and SeeBlue Plus2 pre-stained protein molecular standard were from Invitrogen, CA. Pefabloc was purchased from Pentapharm, Switzerland.

Expression and purification of PAFAHα_1

For protein expression, the plasmid pHis-PAFAHα_1 was transformed into the *E. coli* strain BL21(DE3)-pLysS and colonies were selected on LB agar plates containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. Cells from single drug-resistant colonies were grown overnight in 10 ml LB broth supplemented with the same antibiotics at 37 °C. The next morning, the cells were diluted into 1 L LB broth with appropriate antibiotics in a 2.8 L baffled Fernbach flask. The cells were grown at 37 °C to mid-log phase (A_{600} 0.5–0.6) and the gene was induced with 1 mM IPTG for 3 h. The cells were harvested by centrifugation and the cell pellet was resuspended in 50 ml of 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 1 mM pefabloc, 1 mM PMSF, and 1 μg/ml DNase. The cells were passed three times at 16,000 psi in a Spectronic Unicam 40K FRENCH pressure cell using a FRENCH pressure cell press. The lysate was centrifuged at 13,900*g* for 30 min in a Jouan MR1822 centrifuge to remove the cell debris and unlysed cells. The supernatant containing the PAFAHα_1 was loaded onto a 5 ml bed volume Ni-NTA superflow metal affinity column at a flow rate of 1.0 ml/min. Before loading, the column was pre-equilibrated with buffer A (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl). The unbound protein was then washed with buffer A + 20 mM imidazole until the A_{280} came to the baseline. The proteins were then eluted with buffer A + 250 mM imidazole. The peak fractions were pooled and concentrated using an Amicon concentrator with a YM10 membrane. The concentrated protein was then dialysed against 20 mM Tris–Cl, pH 8.0, for 3 days using 10 kDa MWCO dialysis membrane.

rTEV protease digestion

rTEV protease digestion of the protein was carried out in a total volume of 50 μl containing 1× assay buffer (50 mM Tris–Cl, pH 8.0, 0.5 mM EDTA, and 1 mM DTT), 10 μg PAFAHα_1, and 10 units rTEV protease. Experiments were performed at room temperature and at 30 °C. Sixteen different detergents (Table 1) were added to individual reaction mixture. The reaction was quenched after 6 h by mixing with 4× NuPAGE LDS sample buffer (40% glycerol, 564 mM Tris base, 424 mM Tris–HCl, 8% LDS, 2.04 mM EDTA, 0.4% bromophenol blue, and 0.025% phenol red) and incubating in boiling water bath for 3 min. The degree of rTEV digestion was detected on SDS–PAGE. Each cleavage experiment was repeated four times. The quantity of the digested products was measured qualitatively by visualization and compared with the control experiment that was carried out without any detergent in the reaction. Reactions, identical to the detergent-free control, where no uncut PAFAHα_1 could be detected were referred to as complete (C) digestion. Reactions that yielded uncut PAFAHα_1 were referred to as incomplete (I) digestion.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

SDS–PAGE [19] was performed on a NuPAGE 10% Bis–Tris gel with a Novex Xcell II mini-cell apparatus (Invitrogen, CA). The protein bands were stained by Coomassie brilliant blue R-250 and then destained by boiling the gels in water for 15–20 min.

Results

The objective of this study was to characterize the effects of detergents upon rTEV activity. The soluble test protein used for this study, α_1 subunit of platelet activating factor acetylhydrolase (PAFAHα_1) [18], has previously been cloned in the pHis-Parallel1 vector [20] and expressed in *E. coli*. The fusion protein was expressed from the plasmid pHis-PAFAHα_1 and purified using Ni-NTA column to homogeneity. The untagged protein has a calculated molecular mass of 26 kDa. However, the untagged protein runs as a 29 kDa and the polyhistidine-tagged fusion protein runs with a mass of approximately 32 kDa on SDS–PAGE (data not shown). For control experiments, the rTEV digestion of the PAFAHα_1 was carried out for 3 and 6 h at both room temperature and 30 °C. Analyses of the digested products on SDS–PAGE gel revealed that the rTEV protease cleaves and quantitatively removes the tag from PAFAHα_1 (Fig. 1A, lanes 4–7). No significant difference in digestion was observed when the reactions were carried out for 3 h. vs. 6 h and at room temperature vs. 30 °C. Digestion experiments were then carried out in the presence of 16 different detergents (Table 1) for 6 h. The results of the cleavage experiments, shown in Fig. 1, are summarized in Table 1. Several of the detergents examined, notably APO-10, DDMAB, DODMG, FC-12, HECAMEG, LDAO, OG, and ZW 3–12 inhibit

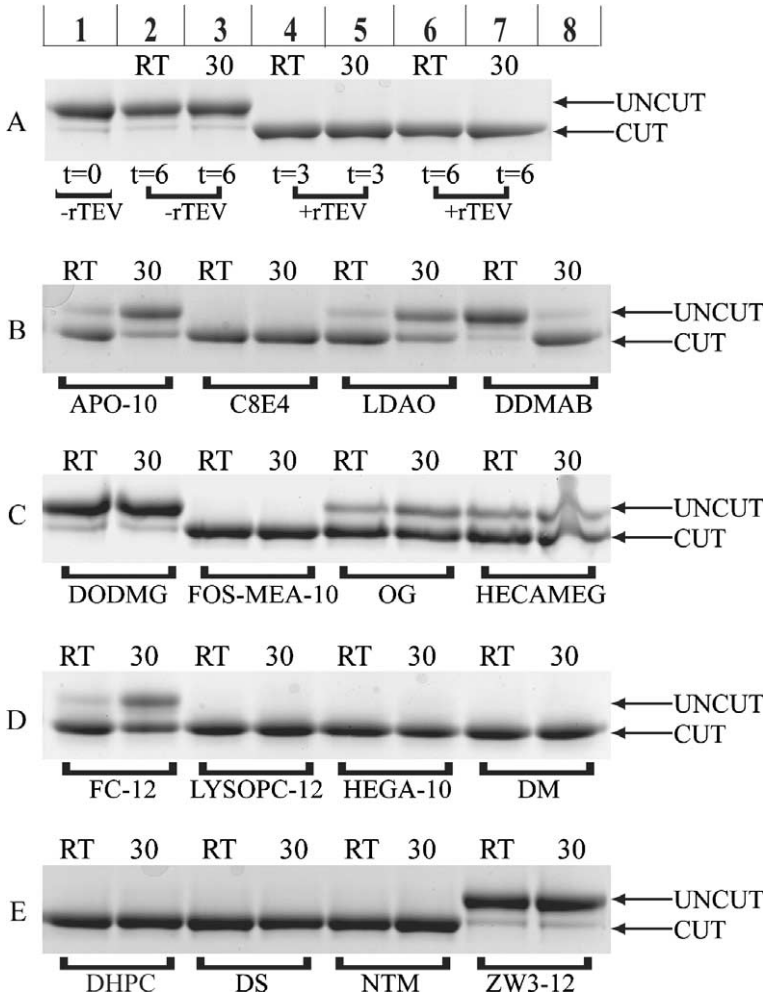

Fig. 1. SDS–PAGE analysis of rTEV protease digestion of PAFAHα_1. Each reaction contains 10 μg PAFAHα_1 and 10 units (as defined in the Life Technologies catalog) rTEV protease. (A)—Control reactions carried out without added detergents. Lanes 1–3: no rTEV added. Lanes 4–7: rTEV at indicated time and temperature. $t = 0$, $t = 3$, and $t = 6$ denote time 0, 3, and 6 h, respectively. (B) to (E)—Reactions performed for 6 h in the presence of different detergents (marked underneath). For all panels: RT = room temperature, 30 = 30 °C, uncut and cut positions of the proteins are marked with arrows, lane positions are marked on top of (A).

rTEV digestion by at least a factor of 2 to 3. No inhibition in digestion was observed in C_8E_4, DHPC, DM, DS, FOS-MEA-10, HEGA-10, LYSOPC-12, and NTM.

Discussion

Inefficient removal of an affinity tag in membrane proteins could occur by steric occlusion of the cleavage site by protein and/or detergent of the protein–detergent complex (PDC), or by an inhibitory effect of detergent upon protease activity. Steric occlusion effects are dependent upon the specific protein and detergent of a particular PDC; thus, these effects need to be assayed on a case-by-case basis. In this study, we sought to answer the more general question: do specific detergents at concentrations used for membrane protein biochemistry inhibit rTEV activity? Our experimental results indicate that eight of sixteen different detergents inhibit rTEV activity, as assayed by less efficient removal of a polyhistidine tag from PAFAHα_1. Detergent concentrations, listed in Table 1, are typical for use in integral membrane protein purification, crystallization, and other biochemical experiments. The specific chainlengths used for each of the given detergent head group 'classes' are typical for uses with integral membrane proteins. We utilized PAFAHα_1 as a test protein because as a soluble protein it will have less interaction with detergents (i.e., we do not expect PAFAHα_1 to form PDCs in the presence of detergents). We observe no simple correlation between the specific properties of a given detergent (charge, CMC, tail chainlength, head group structure, etc.) and its ability to inhibit rTEV protease activity. For example, some uncharged detergents (C_8E_4, DM, DS,

HEGA-10, and NTM) do not inhibit rTEV digestion, while other uncharged detergents (APO-10, HECAMEG, and OG) do inhibit digestion. Some of the detergents with lower CMCs (C_8E_4, DHPC, DM, DS, FOS-MEA-10, HEGA-10, LYSOPC-12, and NTM) do not inhibit rTEV digestion, while other low-CMC detergents (APO-10, DDMAB, DODMG, FC-12, LDAO, and ZW 3–12) do inhibit digestion. Higher CMC values could possibly be a factor in rTEV inhibition. The CMCs of all of the detergents except OG and HECAMEG are between 0.9 and 7.2 mM, whereas OG and HECAMEG have CMCs of 19 and 19.5 mM, respectively, and do inhibit the cleavage. The chainlengths of all of the detergents are between seven and twelve carbons (methylenes and terminal methyl). As evident from Table 1, larger or smaller chainlengths do not play an obvious role in inhibition of rTEV protease activity. LYSOPC-12 has a 12 carbon chainlength and does not inhibit the cleavage; however, DDMAB, DODMG, FC-12, LDAO, and ZW 3–12 possess identical 12 carbon chainlengths but do inhibit the digestion. DHPC has a seven carbon chainlength and does not inhibit the reaction while HECAMEG, also of chainlength seven, does inhibit the digestion. The head group structures of all of the detergents are different and no particular structure of the detergents used is a simple factor in inhibition of rTEV activity.

In the most general case of membrane protein purification and crystallization, three different detergents may be required. One detergent is effective at solubilizing the protein from the cell membrane, another is effective for chromatography, and a third is used for crystallization. Each different detergent entails exchange of the protein into it. Our results indicate that, in this general scenario, a fourth detergent that does not inhibit rTEV protease may be required. Alternatively, certain detergents may need to be avoided during the digestion step, so as to eliminate the possibility of detergent-induced inhibition or inactivation of TEV protease, leading to incomplete digestion and tag removal. While steric occlusion of the protease site within the protein–detergent complex (PDC) is certainly a possibility, avoiding inhibitory detergents will enable this cause to be diagnosed and potentially addressed. Modifications of the protein construct (changing the linker, moving from N to C terminus, etc.) and/or exchanging into a different detergent may overcome this problem.

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