N-Terminal Modification of Proteins with Subtiligase Specificity Variants

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Subtiligase is a powerful enzymatic tool for N-terminal modification of proteins and peptides. In a typical subtiligase-catalyzed N-terminal modification reaction, a peptide ester donor substrate is ligated onto the unblocked N terminus of a protein, resulting in the exchange of the ester bond in the donor substrate for an amide bond between the donor substrate and protein N terminus. Using this strategy, new chemical probes and payloads, such as fluorophores, affinity handles, cytotoxic drugs, and reactive functional groups, can be introduced site-specifically into proteins. While the efficiency of this reaction depends on the sequences to be ligated, a panel of mutants was recently developed that expands the scope of substrate sequences that are suitable for subtiligase modification. This article outlines the steps for applying subtiligase or specificity variants for both site-specific bioconjugation of purified proteins and for global modification of cellular N termini to enable their sequencing by tandem mass spectrometry.

Basic Protocol 1: Subtiligase-catalyzed site-specific protein bioconjugation
Support Protocol 1: Expression and purification of subtiligase-His_6
Support Protocol 2: Subtiligase substrate synthesis
Basic Protocol 2: Subtiligase N terminomics using a cocktail of subtiligase specificity mutants

Keywords: N terminomics • peptide ligase • protein bioconjugation • protein N terminus • proteolysis

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INTRODUCTION

Subtiligase is a variant of the serine protease subtilisin BPN′ that has been engineered to catalyze a ligation reaction between a peptide ester donor substrate and the N-terminal α-amine of a peptide or protein (Abrahmsen et al., 1991). Subtiligase harbors two key mutations compared with the parent protease. Mutation of the catalytic Ser to Cys (S221C) reduces amidase activity to a negligible level but maintains the esterase activity observed in subtilisin (Nakatsuka, Sasaki, & Kaiser, 1987; Neet & Koshland, 1966; Polgar & Bender, 1966; Weeks & Wells, 2019). This enables formation of a thioacyl-enzyme intermediate from the donor peptide ester substrate, which may be hydrolyzed or aminolyzed to form a new peptide bond. Introduction of a second structural mutation, P225A, repositions the

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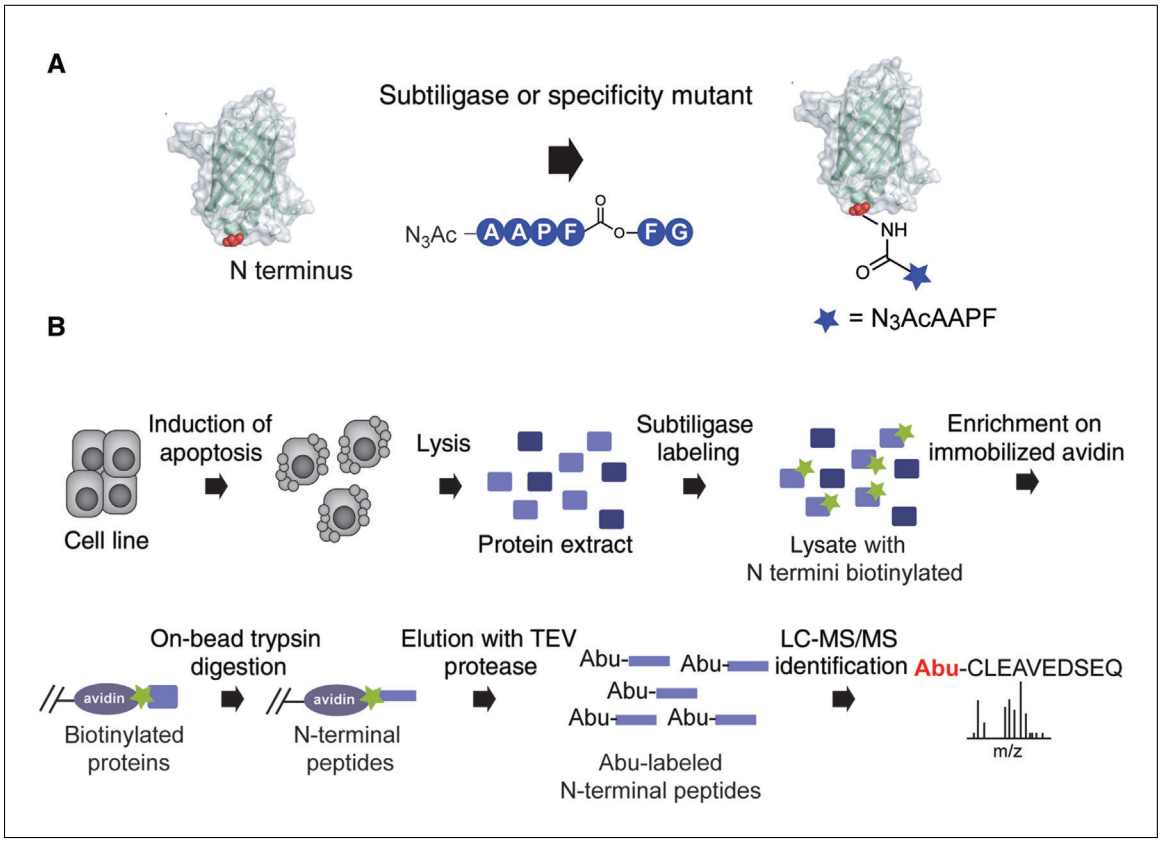

Figure 1 Application of subtiligase and specificity variants for N-terminal modification. (**A**) Subtiligase-catalyzed modification of purified protein N termini. (**B**) Workflow for subtiligase-catalyzed cellular N terminomics. Abu, aminobutyric acid.

larger Cys 221 nucleophile and increases the aminolysis-to-hydrolysis ratio of the intermediate by 50- to 100-fold (Abrahmsen et al., 1991). Based on this engineered catalytic activity, subtiligase is a practical tool for modification of N-terminal α-amines with absolute chemoselectivity over lysine ε-amines. Subtiligase has the advantages that it can be recombinantly expressed in high yield, that it is efficient ($k_{cat}/K_M > 10^5 M^{-1}s^{-1}$), that it has broad sequence specificity (Abrahmsen et al., 1991), and that a large toolbox of specificity variants have been developed to expand its utility (Weeks & Wells, 2018).

This article describes how subtiligase and its variants can be used for site-specific protein modification and for global modification of cellular N termini to enable their sequencing by liquid chromatography tandem mass spectrometry (LC-MS/MS; Fig. 1). The Strategic Planning section describes considerations for subtiligase modification efficiency, including the sequence and structure of the protein(s) to be modified, the selection of an appropriate subtiligase variant or variants, and the reaction conditions used. Basic Protocol 1 describes a method for subtiligase-catalyzed site-specific protein bioconjugation. Support Protocol 1 provides a method for synthesis of peptide ester donor substrates to be used for subtiligase-catalyzed N-terminal modification. Support Protocol 2 provides a method for subtiligase purification. Basic Protocol 2 describes a method for performing subtiligase N terminomics analysis following application of a biological stimulus that induces proteolysis.

STRATEGIC PLANNING

Before beginning Basic Protocol 1, there are several key properties of the target protein that must be considered. Subtiligase requires an unblocked N terminus as its substrate and will not modify acetylated or otherwise blocked N termini (Abrahmsen et al., 1991;

Mahrus et al., 2008). If the target protein has a blocked N terminus and is expressed recombinantly, it can be expressed as a fusion protein that can be cleaved with a protease (such as tobacco etch virus [TEV] protease, PreScission protease, or SUMO protease) to generate an unblocked N terminus that is a subtiligase substrate (Weeks & Wells, 2018). A second consideration is the N-terminal sequence of the target protein. If the sequence is known, the ALPINE (α-Amine Ligation Profiling Informing N-terminal modification Enzyme selection) web application (*https://wellslab.ucsf.edu/alpine*) can be used to select the subtiligase variant that will modify it most efficiently (Weeks & Wells, 2018). If the N-terminal sequence is unknown, a panel of mutants can be screened to determine whether the target protein can be modified. A third consideration is the structure of the target protein N terminus. Subtiligase can modify loops and β-sheets more efficiently than α-helices (Chang, Jackson, Burnier, & Wells, 1994). If the N terminus of the target protein is helical, and the protein can be recombinantly expressed, it can be extended with residues with low helical propensity to enable modification of the N terminus (Chang et al., 1994). Accessibility of the target protein N terminus is also a critical factor. Subtiligase must be able to bind the protein N terminus in an extended conformation to perform efficient modification. If the N terminus is inaccessible, two solutions are possible to enable modification with subtiligase. If the target protein can be refolded after denaturation, detergents or chaotropic agents may be added to the reaction to partially or fully unfold the target protein. This requires use of the stabiligase variant of subtiligase, which contains five stabilizing mutations and retains activity in the presence of 0.1% sodium dodecyl sulfide (SDS) or 4 M guanidinium chloride (GdnHCl; Chang et al., 1994). Stabiligase and its specificity variants are good initial subtiligase variant choices as the five additional stabilizing mutations do not alter substrate specificity. An alternative solution for modifying a protein that is a poor substrate for subtiligase due to N-terminal inaccessibility is to genetically modify the target protein to extend the N terminus such that it becomes accessible to subtiligase (Chang et al., 1994; Weeks & Wells, 2018).

The desired functional group or payload to be introduced should be considered before beginning Support Protocol 2. Support Protocol 2 describes the steps for synthesizing the azide-containing subtiligase substrate N_3Ac-AAPF-glycolate-FG-amide, which is suitable for downstream click chemistry (Weeks & Wells, 2018). This substrate is modular and enables the introduction of any molecule containing an alkyne click chemistry substrate, of which myriad are commercially available. However, certain payloads of interest may not be available as alkyne derivatives. In this case, alternative substrates containing other reactive functional groups can be synthesized. Alternatively, the payload of interest can be directly incorporated into the peptide ester substrate. Supporting Information File 1 provides a spreadsheet for calculating the required amount of reagents for peptide synthesis in the event of changing the scale of the synthesis or the sequence of the subtiligase substrate to be synthesized.

Before beginning Basic Protocol 2, the protease cleavage sites of interest should be considered. Basic Protocol 2 describes the application of a subtiligase mutant cocktail designed to capture the broadest swath of N-terminal sequences that are in the P1′-P2′ positions of protease cleavage sites (Weeks & Wells, 2018). If the protease of interest has a known specificity for the P1′-P2′ residues of its substrates, the ALPINE web application (*https://wellslab.ucsf.edu/alpine*) may be used to design a custom cocktail of subtiligase mutants, which should be used at the same concentrations as those described in the protocol.

Basic Protocol 2 requires the use of a specific subtiligase substrate (TEV ester 6, biotin-EEENLYFQ-Abu-glycolate-R-amide), the sequence of which differs from the sequence described in Support Protocol 2. TEV ester 6 incorporates a biotin affinity handle for

affinity enrichment of subtiligase-modified proteins, a TEV protease cleavage site for selective elution of N-terminal peptides, and an aminobutyric acid (Abu) mass tag for positive identification of peptides that were modified by subtiligase (Shimbo et al., 2012). Although the sequence of this substrate differs from the substrate described in Support Protocol 2, the general method for synthesis is the same. Supporting Information File 1 contains an example spreadsheet in which reagent amounts for synthesis of TEV ester 6 are calculated.

BASIC PROTOCOL 1

SUBTILIGASE-CATALYZED SITE-SPECIFIC PROTEIN BIOCONJUGATION

Based on its ability to catalyze peptide bond formation, subtiligase has been widely applied for site-specific protein bioconjugation at the protein N terminus. Although wild-type subtiligase has broad sequence specificity, not all N-terminal protein sequences are efficient subtiligase substrates. A panel of subtiligase mutants was therefore developed to enable a wider array of N-terminal sequences to be modified. This protocol describes how to apply subtiligase, stabiligase, or variants for modification of the N terminus of a recombinant protein with an azide-bearing peptide. This is achieved by incubating the target protein with subtiligase and a peptide ester substrate. If properly executed, nearly quantitative modification of the target protein is expected (Fig. 2).

Materials

Tricine (e.g., Millipore Sigma, cat. no. T0377)
Peptide ester donor substrate (see Support Protocol 2)
Dimethyl sulfoxide (DMSO; e.g., Fisher Scientific, cat. no. BP231-100) *or* *N,N*-dimethylformamide (DMF; e.g., Sigma-Aldrich, cat. no. 319937)
Target protein to be modified
100 µM subtiligase (see Support Protocol 1)
Ni-NTA wash buffer (optional; see recipe)

pH indicator strips (e.g., EMD Millipore, cat. no. 1095350001)
LC-MS system suitable for intact protein analysis (e.g, Waters Xevo G2-XS coupled to Acquity I-Class LC system)
Analytical high-performance liquid chromatography (HPLC) column suitable for use with LC-MS system (e.g, Waters Acquity Protein BEH C4 column, 2.1-mm inner diameter, 50-mm length, 300-Å pore size, 1.7-µm particle size)

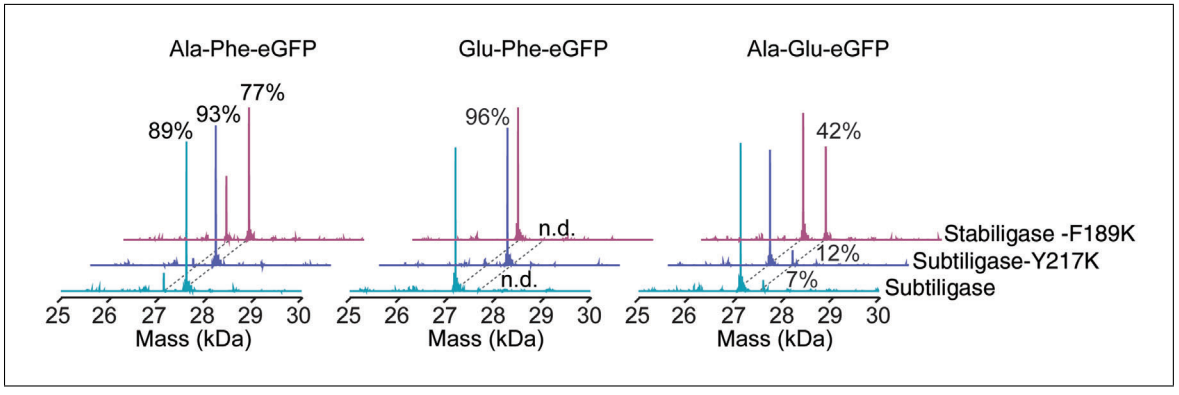

Figure 2 Example data for subtiligase-catalyzed protein bioconjugation. Left: Intact protein mass spectra for subtiligase-catalyzed modification of Ala-Phe-eGFP with N_3Ac-AAPF-glycolate-FG-amide. Ala-Phe-eGFP is efficiently modified by wild-type subtiligase and subtiligase-Y217K and less efficiently modified by stabiligase-F189K. Middle: Glu-Phe-eGFP is not detectably modified by wild-type subtiligase or stabiligase-F189K due to sequence incompatibility but is efficiently modified by subtiligase-Y217K. Right: Ala-Glu-eGFP is not modified efficiently by wild-type subtiligase or subtiligase-Y217K but is modified with moderate efficiently by stabiligase-F189K. n.d., not detectable.

0.5-ml Zeba Spin Desalting Columns, 7K MWCO (e.g., Thermo Fisher Scientific, cat. no. 89882)
0.2-ml HisPur Ni-NTA Spin Columns (optional; e.g., Thermo Fisher Scientific, cat. no. 88224)

Perform bioconjugation reaction

1. Prepare 1 M tricine, pH 8.0.

   A solution of 1 M tricine, pH 8.0, is stable at room temperature for at least 1 year. Other buffers may be used depending on the requirements of the protein to be modified; see the Strategic Planning section for buffer considerations.

2. Prepare 100 mM peptide ester donor substrate (e.g., N_3AcAAPF-glycolate-FG-amide) in DMF or DMSO.

   The peptide ester donor substrate may be dissolved in DMF or DMSO. A typical stock concentration is 100 mM (20×), but this will depend on solubility and the tolerance of protein to be modified for DMSO or DMF. The final peptide ester concentration in the subtiligase reaction mixtures will be 5 mM, and DMSO or DMF concentration is typically kept at <10%.

3. Prepare reaction mixture containing 100 mM tricine, 5 mM peptide ester donor substrate, and 10 to 50 μM target protein to be modified, reserving 0.01 vol. of the total reaction volume for subtiligase. For example, for a 100-μl reaction volume, combine 10 μl of 1 M tricine, 5 μl of 100 mM peptide ester donor substrate stock, the appropriate volume of target protein, and water to a volume of 99 μl.

   Do not add subtiligase yet.

   While a 100-μl reaction volume is typical, the reaction can be scaled up or down as desired, keeping final concentrations the same.

   Use a protease inhibitor cocktail, such as a Roche Complete Protease Inhibitor Tablet, during purification of the target protein.

4. Confirm pH of the reaction mixture is ∼8.0 by testing 1 μl on a strip of pH paper.

   This step is especially important if using a new batch of peptide ester donor substrate or if peptides were made or purified as trifluoroacetic acid (TFA) salts. Peptide ester stock solutions can sometimes be acidic and impact the pH of the reaction mixture. The reaction mixture must be above 7.0 in order for the ligation reaction to work efficiently.

5. Initiate reaction by adding subtiligase to a final concentration of 1 μM from a 100 μM subtiligase stock solution.

6. Allow reaction to proceed for 1 to 2 hr at room temperature.

   If the target protein to be modified is not stable at room temperature, the reaction may be performed at 4°C for 1 to 2 hr. While the reaction may proceed more slowly at 4°C, reaction yield is typically unaffected. Extended reaction times will generally not increase yield as the ester substrate will be consumed within 1 to 2 hr; however, extending the reaction time is not deleterious as the amide bond formed is stable.

Characterize and purify protein bioconjugate

7. Analyze modified protein by LC-MS to analyze the completeness of the reaction.

   Intact protein LC-MS on a Waters Xevo is typically used to assess the completeness of the reaction. However, other assays may be suitable depending on the peptide ester donor substrate that is being ligated to the target protein N terminus. For example, if the protein of interest is being modified with a fluorophore, in-gel fluorescence with appropriate standards may be suitable. If the modification induces a large enough mass shift, a gel shift assay may be used. If an epitope tag is introduced, Western blotting or ELISA may be suitable for detecting the bioconjugate.

8. *Optional:* If modification has occurred but the reaction is not complete, desalt reaction mixture on a 0.5-ml Zeba spin desalting column according to the manufacturer's instructions. To the desalted protein, add peptide ester donor substrate to 5 mM final concentration and subtiligase to 1 μM final concentration. Incubate for 1 to 2 hr. Repeat until the desired reaction completeness is attained.

9. *Optional:* To remove subtiligase from the reaction mixture, pass reaction mixture over a HisPur Ni-NTA spin column equilibrated with Ni-NTA wash buffer according to the manufacturer's instructions. Save the flow-through and the wash.

   If the target protein also contains a His-tag, subtiligase cannot be removed using Ni-NTA affinity chromatography and must be separated from the target protein based on other differences in their properties that are specific to each target protein. It is also possible to irreversibly inactivate subtiligase using thiol-reactive reagents such as N-ethylmaleimide. However, subtiligase inactivation is not necessary, and the effect of such reagents on the target protein must be considered.

10. Remove excess peptide ester, hydrolyzed peptide ester, and exchange the target protein into a suitable buffer for downstream applications.

    Buffer may be exchanged using Zeba spin desalting columns, other types of desalting columns, by concentration and dilution, by dialysis, or using any other method that will separate excess peptide ester and hydrolysis products from the protein of interest.

SUPPORT PROTOCOL 1

EXPRESSION AND PURIFICATION OF SUBTILIGASE-His_6

This protocol describes purification of His-tagged stabiligase (stabiligase-His_6). Stabiligase is expressed under the native subtilisin promoter in *Bacillus subtilis* as a pre-pro-enzyme that is secreted into the medium (Fig. 3A). The *B. subtilis* strain BG2864, which has the genes encoding subtilisin and neutral protease deleted (Δ*aprE*, Δ*nprE*), is used for expression. After expression, cells are removed by centrifugation, and subtiligase is precipitated from the medium using ethanol. The precipitated protein is dissolved in Ni-NTA wash buffer and purified by Ni-NTA affinity chromatography. If executed properly, this protocol is expected to yield the subtiligase variant at >95% purity (Fig. 3B).

Materials

B. subtilis BG2864 (ATCC #40796)
LB agar plates (see recipe)
2× YT medium (e.g., Fisher Scientific, cat. no. BP246710)
Medium A (see recipe)
Stabiligase expression plasmid, where subtiligase can be replaced with desired subtiligase or stabiligase specificity variant (pBS42-pre-pro-stabiligase-His_6; available upon request from J.A.W.)

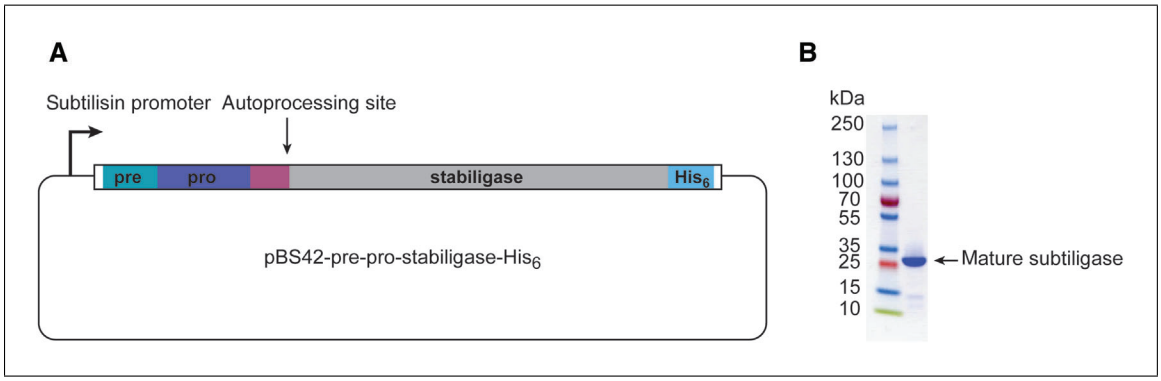

Figure 3 Purification of stabiligase-His_6. (**A**) Schematic of the expression plasmid for producing stabiligase-His_6 in *Bacillus subtilis*. (**B**) SDS-PAGE analysis of purified subtiligase-His_6.

Medium B (see recipe)
Chloramphenicol
80% (v/v) glycerol
1 M $CaCl_2$
95% ethanol (e.g., VWR, cat. no. 89125)
HisPur Ni-NTA resin (e.g., Thermo Fisher Scientific, cat. no. 88221)
Ni-NTA wash buffer (see recipe)
Ni-NTA elution buffer (see recipe)
Subtiligase storage buffer (see recipe)
10-well 4% to 20% mini-gel, suitable for 2- to 400-kDa proteins (e.g., Mini-PROTEAN TGX Precast Protein Gel, Bio-Rad, cat. no. 4561094 or similar)
Protein molecular weight marker, suitable for 10- to 250-kDa proteins (e.g., PageRuler Plus Prestained Protein Ladder, Thermo Fisher, cat. no. 26619 or similar)

37°C incubator with variable shaking
Disposable inoculating loop
Centrifuge
500- and 2000-ml baffled Erlenmeyer flasks
Spectrophotometer
14-ml disposable round-bottom culture tubes
4-L beaker
2.5-ml Thermo HisPur Ni-NTA spin column
50-ml conical tubes
Rotisserie mixer
Dialysis membrane, 10,000 MWCO or lower

Additional reagents and equipment for SDS-PAGE (see Current Protocols article: Gallagher, 2012)

Transform Bacillus subtilis

1. Streak *B. subtilis* BG2864 on an LB agar plate, and grow overnight at 37°C.
2. Inoculate 5 ml of 2× YT medium with a single colony using a disposable inoculating loop. Grow overnight at 37°C with shaking at 200 to 250 rpm.

   A disposable inoculating loop is recommended because B. subtilis may survive mild flaming on a reusable loop.
3. Centrifuge 1 ml overnight culture 20 min at 4000 × *g*, room temperature. Resuspend in 1 ml medium A, and add cell suspension to 50 ml medium A in a 500-ml baffled Erlenmeyer flask. Grow at 37°C with shaking at 200 rpm.
4. Measure optical density at 600 nm (OD_{600}) every 20 min, and plot a growth curve. Note the time when growth begins to depart from log phase. Grow for an additional 90 min from the point of departure from log phase.

   There will be a brief lag phase, followed by log phase growth (see example growth curve in Fig. 4).

   The culture is now at peak competence at the end of this step. Total growth time is usually 4 to 6 hr.
5. When cells are approaching peak competence, put 1 to 2 μg concatameric plasmid DNA (pBS42-pre-pro-stabiligase-His_6) into the bottom of a 14-ml disposable round-bottom culture tube. Prewarm medium B to 37°C.

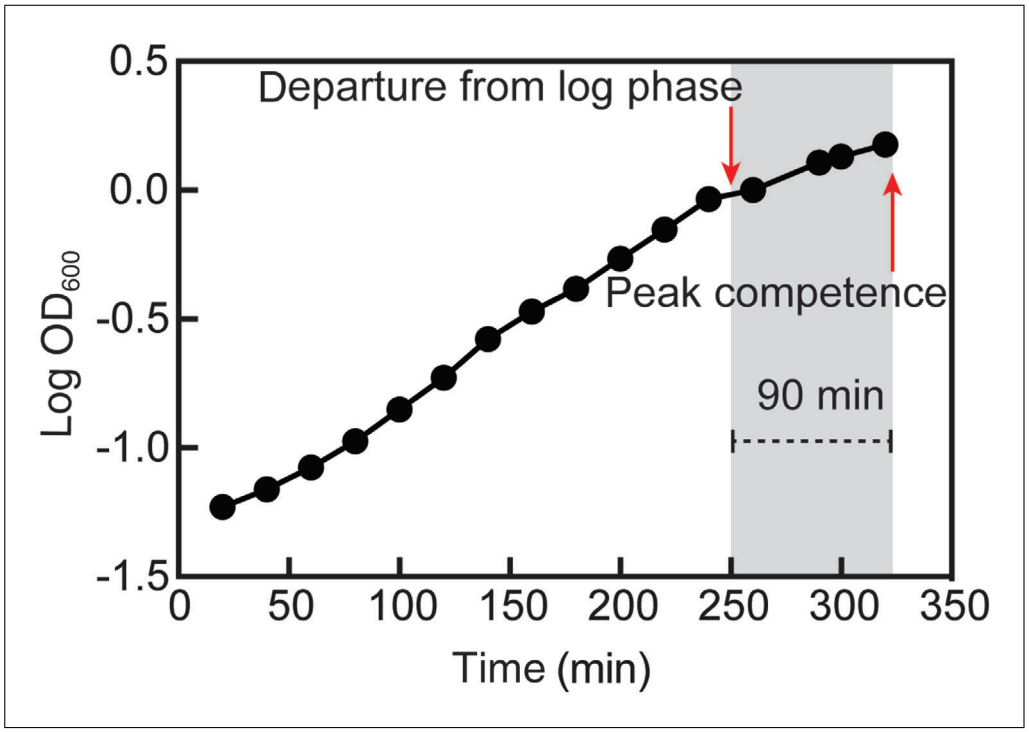

Figure 4 Example growth curve for *Bacillus subtilis.* Red arrows mark departure from log phase and the expected point of peak competence.

Plasmid DNA used for B. subtilis transformation must be concatemeric and should be purified from a recA$^+$ Escherichia coli strain such as ER1821 or JM101 (e.g., New England Biolabs, cat. no. E4102). Concatameric DNA can be prepared using a Qiagen miniprep kit according to the manufacturer's protocol, omitting the optional buffer PB wash step.

6. When cells reach peak competence, dilute 0.5 ml competent cell culture into 5 ml medium B. Add 300 µl mixture into tubes containing plasmid DNA from step 5.
7. *Optional:* Grow at 37°C for 3 hr with shaking at 200 rpm.
8. Plate the entire transformation on an LB agar plate with 5 µg/ml chloramphenicol. Grow overnight to up to 24 hr at 37°C.

The concentration of chloramphenicol (5 µg/ml) used for B. subtilis growth is lower than that used for E. coli. Depending on the time of day that the transformation is plated, colonies may be small and difficult to see the next morning. Allow a full 24 hr at 37°C to see robust colony growth.

Express subtiligase-His$_6$

9. Inoculate 5 ml of 2× YT medium containing 12.5 µg/ml chloramphenicol with a single colony from the plate. Grow overnight at 37°C with shaking at 200 rpm.
10. *Optional:* Prepare a glycerol stock of transformed *B. subtilis* BG2864 for use in future expressions by mixing 250 µl of 80% (v/v) glycerol with 1.5 ml overnight culture. Store at −80°C.
11. Check OD_{600} of the overnight culture.

The culture may be quite dense and may require 1:10 dilution to obtain an accurate OD_{600} reading.

12. Prepare 1.5 L of 2× YT medium supplemented with 12.5 µg/ml chloramphenicol (from a 12.5 mg/ml stock solution) and 5 mM $CaCl_2$ (from a 1 M stock solution)

in a baffled flask. Inoculate medium to OD_{600} of 0.03 to 0.05. Grow for 20 to 24 hr with shaking at 200 rpm.

Subtiligase will be expressed and secreted into the medium in its mature, processed form.

13. Centrifuge 10 min at 4000 × *g*, 4°C, to remove cells from the medium, which contains subtiligase.

After stopping shaking, work quickly to minimize cell lysis, which will lead to higher levels of contaminating protein in the medium.

Purify subtiligase-His_6

14. Pour off 1 L supernatant into a 4-L beaker. Add 3 vol. ice-cold 95% ethanol (3 L). Stir at 4°C for 30 to 60 min.

Subtiligase will precipitate from the medium.

15. Centrifuge 30 min at 4000 × *g*, 4°C, to collect ethanol precipitate. Discard supernatant. Invert centrifuge bottles onto paper towels, and allow pellets to dry for 15 min at room temperature.

16. Prepare a 2.5-ml Thermo HisPur Ni-NTA column for subtiligase-His_6 binding. Wash resin with water, and then equilibrate with Ni-NTA wash buffer (5 to 10 column vol.).

17. Resuspend ethanol-precipitated pellet in 20 ml Ni-NTA wash buffer. After pellet is resuspended well, centrifuge 10 min at 4000 × *g*, 4°C. Keep subtiligase-containing supernatant.

This will yield a gelatinous pellet that should be discarded.

18. Transfer prepared Ni-NTA resin into a 50-ml conical tube. Centrifuge 3 min at 500 × *g*, 4°C, and discard supernatant. Add subtiligase-containing supernatant from step 17 to the resin, and incubate at 4°C for 1 hr on a rotisserie mixer or similar.

19. Collect resin by centrifuging 10 min at 500 × *g*, 4°C, and remove supernatant. Resuspend resin in Ni-NTA wash buffer, and transfer to a plastic column.

20. Wash column with 20 column vol. Ni-NTA wash buffer.

21. Elute subtiligase-His_6 with 3 column vol. Ni-NTA elution buffer. Monitor absorbance of the eluate at 280 nm ($\varepsilon_{subtiligase} = 31{,}400\ M^{-1}cm^{-1}$) to ensure that all protein is collected.

22. Dialyze eluted protein into subtiligase storage buffer.

23. Following dialysis, measure absorbance of the protein solution at 280 nm ($\varepsilon = 31{,}400\ M^{-1}cm^{-1}$). Adjust concentration to 100 μM by concentrating or by diluting with subtiligase storage buffer. Make 100-μl aliquots. Flash freeze and store at −80°C.

24. Evaluate purity of the protein by SDS-PAGE. Load 5 μg purified protein into one well of a 10-well 4% to 20% mini-gel. In an adjacent well, load 5 μl protein molecular weight standard. Run gel according to the manufacturer's instructions.

An example gel is shown in Figure 3B. Typically purity will be >95%.

Aliquots are single use and may be made at smaller volume if desired.

SUBTILIGASE SUBSTRATE SYNTHESIS

Subtiligase requires a peptide ester as its substrate. This protocol describes how to synthesize N_3Ac-AAPF-glycolate-FG-amide (Fig. 5) on a 2-g scale using fluorenylmethoxycarbonyl (Fmoc) solid-phase peptide synthesis (Suich, Ballinger, Wells, & DeGrado, 1996). This peptide ester substrate is used to introduce an azide into the target protein. The synthetic protocol can be adjusted for different sequences and scales. To adjust the scale, use Supporting Information File 1 to calculate amounts of amino acid, 1-hydroxybenzotriazole hydrate (HOBt), and *N,N′*-diisopropylcarbodiimide (DICI) by filling in the fields in blue. If a different sequence will be synthesized, note that the only special steps are those for making the ester bond (steps 10 to 17); otherwise, standard Fmoc-*tert*-butyl (Fmoc/tBu) chemistry can be used to make any sequence.

The exact reagent volumes and amounts can be found in Supporting Information File 1.

Materials

Rink Amide AM resin, 100 to 200 mesh, 0.46 mmol/g (e.g., Millipore Sigma, cat. no. 855130)
Dichloromethane
DMF (e.g., Sigma-Aldrich, cat. no. 319937)
Methanol
20% (v/v) 4-methylpiperidine (e.g., Sigma-Aldrich, cat. no. M73206) in DMF
HOBt (e.g., Chem-Impex, cat. no. 24755)
Fmoc-Gly-OH (e.g., Chem-Impex, cat. no. 02416)
DICI (e.g., Sigma-Aldrich, cat. no. D125407)
Fmoc-Phe-OH (e.g., Chem-Impex, cat. no. 02443)
Acetoxyacetic acid (e.g., Sigma-Aldrich, cat. no. 302341)
2.5 M hydrazine monohydrate (e.g., Sigma-Aldrich, cat. no. 207942) in DMF
4-(dimethylamino)pyridine (DMAP; e.g., Sigma-Aldrich, cat. no. 107700)
Fmoc-Pro-OH (e.g., Chem-Impex, cat. no. 02448)
Fmoc-Ala-OH (e.g., Chem-Impex, cat. no. 02369)
2-azidoacetic acid (e.g., Sigma-Aldrich, cat. no. 763470)
TFA (e.g., Sigma-Aldrich, cat. no. T6508)
Triisopropylsilane (TIPS; e.g., Sigma-Aldrich, cat. no. 233781)
Diethyl ether (e.g., Sigma-Aldrich, cat. no. 676845)
Acetonitrile (e.g., Fisher Scientific, cat. no. A998) or DMSO (e.g., Fisher Scientific, cat. no. BP231-100)

100-ml peptide synthesis vessel with 24/40 inner joint (e.g., ChemGlass, cat. no. CG-1866-04)
Laboratory rocker

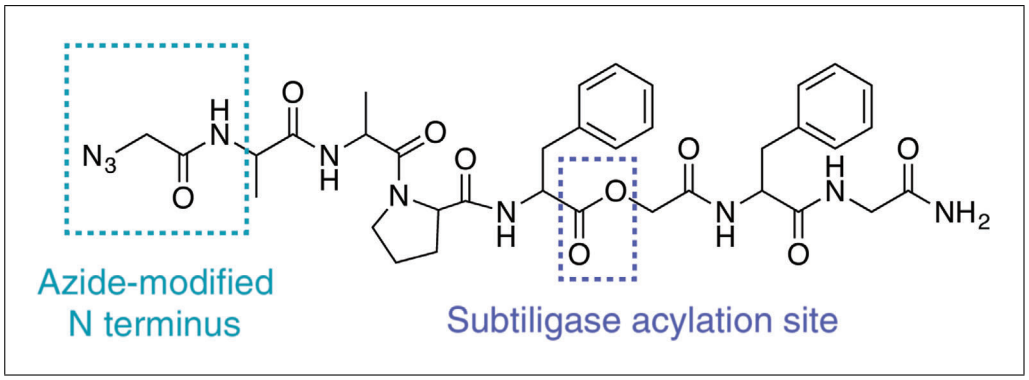

Figure 5 Chemical structure of N_3AcAAPF-glycolate-FG-amide. Key features are boxed with dotted lines.

Vacuum line equipped with solvent trap
500-ml single-neck flat-bottom flask with 24/40 outer joint (e.g., ChemGlass, cat. no. CG-1500-04)
Glass scintillation vial
Round-bottom flask
Rotary evaporator
50-ml conical centrifuge tube
Centrifuge
Analytic HPLC system
Semipreparative or preparative HPLC system
Vacuum centrifuge
Lyophilizer
LC-MS system suitable for analysis of small molecules and peptides

Perform resin swelling

1. Weigh out 2 g Rink Amide AM resin (0.46 mmol/g) into a peptide synthesis vessel. Add 20 ml dichloromethane. Place on a rocker for 30 min.

   The substitution of the resin varies by lot and is typically 0.4 to 0.6 mmol/g. This value will be needed to calculate reagent amounts in subsequent steps.

2. Remove dichloromethane by vacuum filtration into a 500-ml flat-bottom flask. Perform the following wash steps, removing solvent by vacuum filtration between each step: 20 ml DMF, 20 ml DMF, 20 ml DMF, 20 ml methanol, 20 ml dichloromethane, 20 ml DMF, 20 ml DMF, and 20 ml DMF.

   Use a very small amount of vacuum grease on the inner joint of the peptide synthesis vessel to keep it from getting stuck inside the flat-bottom flask when placed under vacuum.

Perform deprotection and coupling cycle

3. Deprotect resin by adding 20 ml of 20% (v/v) 4-methylpiperidine in DMF. Place reaction vessel on a rocker for 30 min.
4. Remove 4-methylpiperidine by vacuum filtration.
5. Wash resin with 20 ml DMF. Repeat four additional times.
6. Couple Fmoc amino acid to the resin. Dissolve 5 equiv. (relative to substitution of resin) HOBt (4.6 mmol, 621 mg) in 10 ml DMF in a glass scintillation vial. Dissolve 5 equiv. Fmoc-Gly-OH (4.6 mmol, 1.4 g) in 10 ml DMF in a separate vial. Add both solutions to the peptide synthesis vessel. Add 5 equiv. DICI (4.6 mmol, 580 mg, 0.72 ml). Place reaction vessel on a rocker, and incubate for 1 hr.
7. Remove solvent by vacuum filtration.
8. Wash resin with 20 ml DMF. Repeat four additional times.
9. Repeat steps 3 through 8 with Fmoc-Phe-OH (1.8 g).

Introduce glycolic acid moiety

10. Deprotect resin by adding 20 ml of 20% (v/v) 4-methylpiperidine. Place reaction vessel on a rocker for 30 min.
11. Remove 4-methylpiperidine by vacuum filtration.
12. Wash resin five times with 20 ml DMF.
13. Dissolve 5 equiv, (relative to substitution of resin) HOBt (4.6 mmol, 621 mg) in 10 ml DMF in a glass scintillation vial. Dissolve 5 equiv. acetoxyacetic acid (4.6 mmol, 543 mg) in 10 ml DMF in a separate vial. Add both solutions to the

peptide synthesis vessel. Add 5 equiv. DICI (4.6 mmol, 580 mg, 0.72 ml). Place reaction vessel on a rocker, and incubate for 1 hr.

14. Remove solvent by vacuum filtration.

15. Wash resin with 20 ml DMF. Repeat four additional times.

16. Remove acetate protecting group. Prepare 20 ml of 2.5 M hydrazine monohydrate (2.4 ml hydrazine monohydrate in 20 ml DMF). Add solution to the resin, and place reaction vessel on a rocker. Allow reaction to proceed for 12 to 16 hr.

Form ester linkage

17. Dissolve 1 mol % DMAP (0.01 mmol, 1.1 mg) in 10 ml DMF in a glass scintillation vial. Dissolve 5 equiv. Fmoc-Phe-OH (4.6 mmol, 1.8 g) in 10 ml DMF in a separate vial. Add both solutions to the peptide synthesis vessel. Add DICI to 1 M final concentration (3.1 ml). Allow reaction to proceed for 1 hr.

The reaction conditions for formation of the ester linkage are different than those used in the standard deprotection and coupling cycle. HOBt is omitted, DMAP is used as a catalyst, and DICI is used at a higher concentration.

Perform deprotection and amino acid coupling following ester formation

18. Repeat steps 3 through 8 to couple the following amino acids: Fmoc-Pro-OH (4.6 mmol, 1.6 g); Fmoc-Ala-OH (4.6 mmol, 1.4 g); and Fmoc-Ala-OH (4.6 mmol, 1.4 g).

Perform azidoacetylation of peptide ester N terminus

19. Deprotect peptide ester by adding 20 ml of 20% (v/v) 4-methylpiperidine. Place reaction vessel on a rocker for 30 min.

20. Dissolve 5 equiv. azidoacetic acid (0.5 g, 0.34 ml) in 10 ml DMF. Dissolve 5 equiv. HOBt (4.6 mmol, 621 mg) in10 ml DMF in a separate vial. Add both solutions to the peptide synthesis vessel. Add 5 equiv. DICI (4.6 mmol, 580 mg, 0.72 ml). Place reaction vessel on a rocker, and incubate for 1 hr.

Cleave peptide from resin

21. Wash resin with 20 ml DMF. Repeat four additional times.

22. Wash resin with 20 ml dichloromethane. Repeat four additional times. After removing the last wash, air dry the resin.

23. Transfer resin to a round-bottom flask. Add 50 ml cleavage cocktail (47.5/1.25/1.25 TFA/water/TIPS). Swirl mixture gently, and allow cleavage to proceed for 90 min.

The peptide sequence described in this protocol does not require any side chain protecting groups. If a different sequence is synthesized that requires side chain protecting groups, they should be cleavable by TFA so that they can be removed at the same time the peptide is cleaved from the resin.

24. Filter mixture to remove the resin and collect filtrate. Concentrate to ~5 to 10 ml by blowing air over the solution or by using a rotary evaporator.

25. Add concentrated filtrate to 10 vol. diethyl ether in 50-ml conical centrifuge tubes. Allow peptide to precipitate for 10 min. Isolate precipitated peptide by centrifugation 10 min at 2000 × *g*, 4°C.

26. Remove supernatant. Resuspend pellet in diethyl ether to wash the crude peptide. Centrifuge 10 min at 2000 × *g*, 4°C, to collect the precipitated peptide.

Characterize and purify crude peptide

27. Dissolve a small amount of crude peptide in acetonitrile or DMSO. Inject onto an analytical HPLC system to assess purity.

    Purity may be evaluated based on the chromatographic trace from absorbance at 210 nm or from an evaporative light scattering detector (ELSD).

28. *Optional:* Dissolve 100 mg crude peptide in acetonitrile or DMSO. Inject onto a semipreparative or preparative HPLC system for purification (buffer A: 0.1% aqueous TFA; buffer B: acetonitrile/0.1% TFA). Collect fractions and keep those corresponding to the main peak. Remove organic solvent in a vacuum centrifuge or on a rotary evaporator, and then lyophilize to remove the aqueous solution.

    N_3Ac-AAPF-glycolate-FG-amide typically elutes around 70% buffer B.

    Fractions should be assessed for purity using an analytical HPLC method before combining. Alternative compositions of buffer B such as methanol/0.1% TFA may be used.

29. Confirm identity of the peptide by MS.

    For N_3Ac-AAPF-glycolate-FG-amide, the expected [M+H^+] is 749.3.

    Liquid chromatography electrospray ionization MS (LC-ESI-MS) is typically used; however, matrix-assisted laser desorption/ionization MS (MALDI-MS) is also suitable.

SUBTILIGASE N TERMINOMICS USING A COCKTAIL OF SUBTILIGASE SPECIFICITY MUTANTS

BASIC PROTOCOL 2

Global sequencing of unblocked protein N termini can provide key insights into how proteolytic modification impacts protein function (Griswold et al., 2019; Kleifeld et al., 2010; Mahrus et al., 2008). Because wild-type subtiligase does not efficiently modify all protein N-terminal sequences, a cocktail of subtiligase mutants was designed to maximize modification of all possible N-terminal dipeptide sequences. This enables enrichment and purification of a large number of N-terminal and semitryptic peptides from cell lysate for sequencing by LC-MS/MS. This protocol describes the steps for global sequencing of protein N termini following induction of apoptosis by the topoisomerase II inhibitor etoposide. This is achieved by adding the subtiligase cocktail to a cell lysate from etoposide-treated cells, along with a subtiligase substrate (TEV ester 6; Fig. 6) containing an N-terminal biotin for affinity enrichment, a TEV protease cleavage site for selective elution of N-terminal peptides, and an Abu tag for positive identification of subtiligase-modified peptides. The N-terminal peptides are then enriched and analyzed by LC-MS/MS, enabling the identification of the exact positions of protease cleavage sites. If properly executed, identification of hundreds of N termini from an untreated lysate and hundreds to thousands of N termini from an etoposide-treated lysate is expected (Fig. 7A). In the etoposide-treated lysate, hundreds of N termini are expected

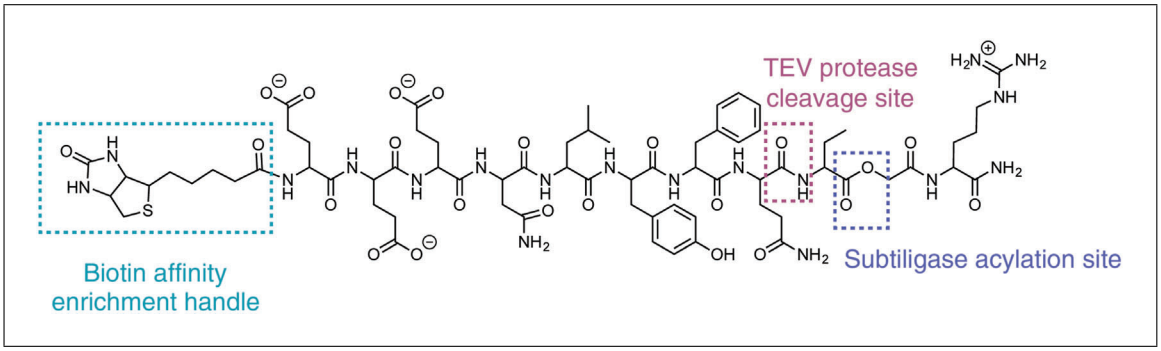

Figure 6 Chemical structure of TEV ester 6. Key features are boxed with dotted lines. TEV, tobacco etch virus.

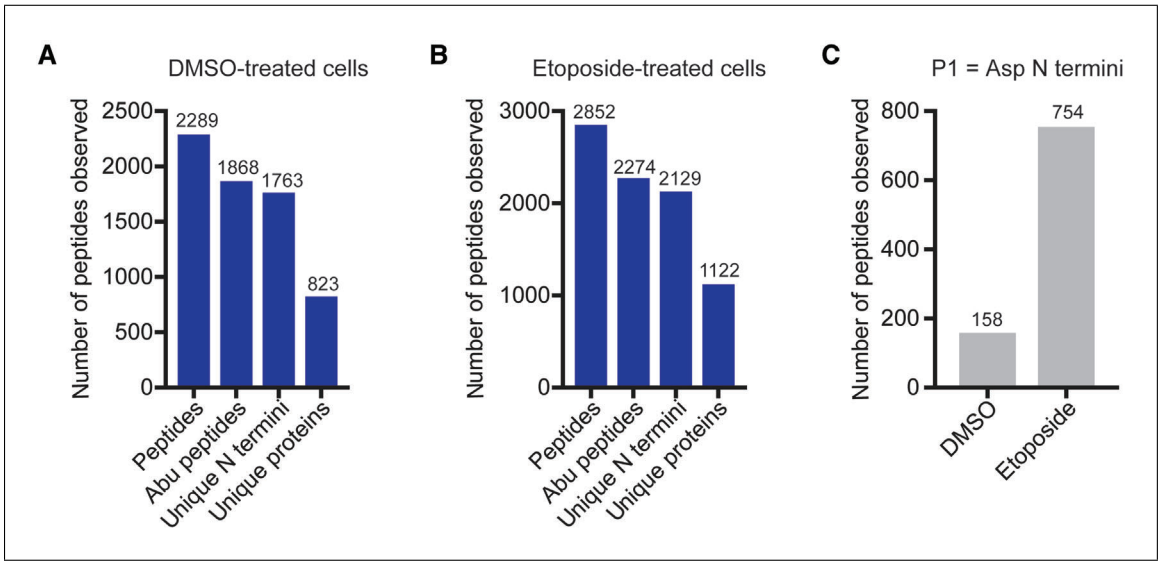

Figure 7 Example data for a subtiligase cocktail N terminomics experiment. Number of peptides identified in the (**A**) DMSO-treated control and (**B**) etoposide-treated sample. (**C**) Peptides with aspartate in the P1 position for DMSO- and etoposide-treated samples. Proteolytic cleavages following aspartate are increased following etoposide treatment because caspase cleavages are induced. Abu, aminobutyric acid; DMSO, dimethyl sulfoxide.

to follow an aspartate residue, the sequence signature of caspase cleavages (P1 = D) that occurs during apoptosis (Fig. 7B).

Materials

Jurkat E6-1 cells (ATCC #TIB-152)
RPMI-1640 (e.g., GE Life Sciences, cat. no. SH30027)
50 mM etoposide (e.g., Sigma-Aldrich, cat. no. E1383) in DMSO
DMSO (e.g., Fisher Scientific, cat. no. BP231-100)
Phosphate-buffered saline (PBS; e.g., GE Life Sciences, cat. no. SH30256)
CellTiter Glo Luminescent Cell Viability Assay kit (optional; e.g., Promega)
4× lysis buffer (see recipe)
0.5 M ethylenediaminetetraacetic acid (EDTA)
100 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF; e.g., Sigma-Aldrich, cat. no. A8456) in DMSO
100 mM phenylmethanesulfonyl fluoride (PMSF; e.g., Sigma-Aldrich, cat. no. P7626) in isopropanol
1 M tris(2-carboxyethyl)phosphine hydrochloride (TCEP; e.g., Sigma-Aldrich, cat. no. C4706)
500 mM iodoacetamide (e.g., Sigma-Aldrich, cat. no. I1149)
1 M dithiothreitol (DTT; e.g., GoldBio, cat. no. DTT100)
Triton X-100 (e.g., Sigma-Aldrich, cat. no. T8787)
25 mM TEV ester 6 (see Strategic Planning, Support Protocol 2, and Supporting Information File 1)
Subtiligase cocktail (stabiligase, stabiligase-F189S/Y217K, stabiligase-F189D, and stabiligase-Y217D/M222A)
Acetonitrile (e.g., Fisher Scientific, cat. no. A998)
4 and 8 M GdnHCl (e.g., Chem-Impex, cat. no. 00152)
95% ethanol (e.g., VWR, cat. no. 89125)
High-Capacity NeutrAvidin Agarose resin (e.g., Thermo Fisher, cat. no. 29202)
100 mM ammonium bicarbonate, pH 8.0 (e.g., Sigma Aldrich, cat. no. 09830)
Sequencing-grade modified trypsin (e.g., Promega, cat. no. V5113)
TEV protease (purchased from commercial sources or purified in-house)

5% (v/v) TFA (e.g., Sigma-Aldrich, cat. no. T6508)
Formic acid (e.g., Fisher Scientific, cat. no. A117)

Cell culture flasks
37°C, 5% CO_2 cell culture incubator
Centrifuge
15- and 50-ml conical tubes
Probe ultrasonicator
Microcentrifuge
0.6- and 1.5-ml low-binding tubes (e.g., Axygen, cat. nos. MCT-060-L-C and MCT-150-L-C, respectively)
Heating block
pH indicator strips (e.g., EMD Millipore, cat. no. 1095350001)
Rotating mixer
1-ml snap-cap spin columns (e.g, Thermo Fisher Scientific, cat. no. 69725)
Vacuum manifold
Vacuum concentrator
C18 spin desalting tips (e.g., Thermo Fisher Scientific, cat. no. 84850)
LC-MS/MS system suitable for proteomics (e.g., Thermo Dionex UltiMate 3000 RSLCnano LC system equipped with an Acclaim PepMap RSLC column [75 μm × 15 cm, 2-μm particle size, 100-Å pore size; Thermo Scientific] coupled to a Thermo Q-Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer)
Computer running MSconvert and Protein Prospector software (available at *http://prospector.ucsf.edu*)

Induction of apoptosis and cell harvest

1. Grow two flasks of Jurkat E6-1 cells to a density of 8×10^5 cells per ml in a total volume of 300 ml RPMI-1640.

2. Induce apoptosis by adding 300 μl of 50 mM etoposide in DMSO (final concentration 50 μM) to one flask. Add an equal volume of DMSO to the other flask as a no-etoposide control. Grow cells for 10 hr at 37°C.

   An incubation time of 10 hr with etoposide usually results in 40% to 50% cell viability. If desired, monitor cell viability using the Promega CellTiter Glo Luminescent Cell Viability Assay kit or another cell viability assay. Cells should be harvested when they reach ∼50% viability. Other apoptosis inducers, such as staurosporine, may be used but require different incubation times.

3. Harvest cells by centrifugation 5 min at 300 × *g*, room temperature. Resuspend in 15 ml PBS, and transfer to a 15-ml conical tube. Centrifuge 5 min at 300 × *g*, room temperature, to pellet cells.

   If an adherent cell line is used, cells should be harvested using methods that do not introduce protease activity, such as incubation with versene or scraping.

Cell lysis

4. Prepare 1 ml of 4× lysis buffer. Immediately before use, add 5 μl of 0.5 M EDTA, 5 μl of 100 mM AEBSF (dissolved in DMSO), and 5 μl of 100 mM PMSF (dissolved in isopropanol).

   AEBSF and PMSF are protease inhibitors that will not interfere with subtiligase function.

5. Add 500 μl of 4× lysis buffer to each cell pellet. Use probe ultrasonication (5 s on and 1 s off, 20% amplitude, 10 cycles; repeat as needed) to lyse cells completely.

   Following ultrasonication, the lysate should look almost completely clear. If intact cells remain, use more lysis buffer, and scale the subsequent steps of the protocol accordingly.

6. Centrifuge lysate 10 min at maximum speed, 4°C, in a benchtop microcentrifuge. Transfer supernatant to a new tube and discard pellet.

   This lysis method usually does not produce a visible pellet.

Protein reduction and alkylation

7. Add 1 M TCEP to a final concentration of 5 mM. Heat sample at 95°C for 15 min. Allow sample to cool to room temperature.

   The 1 M TCEP stock should be carefully neutralized to avoid protein precipitation upon addition to the sample. The sample may be heated in a heating block or placed in a beaker of boiling water for 15 min. TCEP treatment reduces all cysteine residues in the sample in preparation for iodoacetamide treatment.

8. Prepare 500 mM iodoacetamide (stock solution). Add iodoacetamide to the samples to a final concentration of 10 mM. Wrap samples in aluminum foil to shield from light, and incubate for 1 hr at room temperature.

   The iodoacetamide stock solution should be prepared fresh daily. Iodoacetamide treatment covalently modifies free cysteines in the samples, preventing them from forming undesired disulfide bonds.

9. Add 1 M DTT to a final concentration of 25 mM to quench the remaining iodoacetamide. Vortex briefly.

   This step is critical as subtiligase will be inhibited if there is free iodoacetamide in the sample.

10. Add Triton X-100 to a final concentration 2.5% (v/v) to form micelles with SDS.

    Addition of Triton X-100 leads to formation of micelles and is critical to enable subtiligase to function in the presence of SDS.

11. Dilute sample so that lysis buffer is at a final concentration of $1\times$. For example, if 500 µl lysis buffer was used, add distilled deionized water to a final volume of 2 ml.

Subtiligase labeling of cellular N termini

12. Add 25 mM TEV ester 6 to a final concentration of 2.5 mM (200 µl for a 2-ml reaction volume). Test pH of the reaction mixture using pH paper to ensure that the pH remains >7.

    This step is especially important if using a new batch of TEV ester 6 or if the peptide was made or purified as a TFA salt. The reaction mixture must be above pH 7 in order for the ligation reaction to work efficiently.

13. Remove a 50-µl aliquot of the reaction mixture, and save at -20°C as a prelabeling sample for concentration determination and Western blotting.

14. Make subtiligase mutant cocktail. In a separate 1.5-ml tube, mix 20 µl of each of the subtiligase mutants to be used for labeling (or 0.01 vol. of the total reaction mixture volume).

15. Add mutant cocktail to the reaction mixture. Incubate for 1 to 2 hr at room temperature.

16. Remove a 50-µl aliquot of the reaction mixture, and save at -20°C as a postlabeling sample for concentration determination and Western blotting.

Removal of excess peptide ester substrate

17. Precipitate sample by adding it dropwise to 10 vol. acetonitrile in a 50-ml conical tube. Incubate on ice for 15 min or at 4°C for up to 16 hr.

    Acetonitrile is used for precipitation because it precipitates protein effectively, but excess TEV ester 6 remains in solution. This step represents a good pause point.

18. Centrifuge 30 min at 4000 × *g*, 4°C, to pellet the precipitate. Discard supernatant. Invert tube onto a paper towel, and allow pellet to dry for 15 min.

19. Add 500 µl of 8 M GdnHCl to solubilize the pellet. Shake gently for 30 to 60 min to allow the pellet to dissolve. If pellet does not dissolve completely, use probe ultrasonication (5 s on and 1 s off, 20% amplitude) until completely dissolved.

20. Add 500 µl water to dilute GdnHCl to 4 M.

21. Precipitate protein a second time by adding the sample to 10 vol. ice-cold 95% ethanol. Incubate at −80°C for 2 hr to overnight.

    Ethanol precipitation helps to remove residual SDS in the sample. This step is a potential pause point.

22. Centrifuge 30 min at 4000 × *g*, 4°C, to pellet the precipitate. Discard supernatant. Invert tube onto a paper towel, and allow pellet to dry for 15 min.

23. Add 500 µl of 8 M GdnHCl to solubilize the pellet. Shake gently for 30 to 60 min to allow the pellet to dissolve. If pellet does not dissolve completely, use probe ultrasonication (5 s on and 1 s off, 20% amplitude) until completely dissolved.

24. Add 500 µl water to dilute GdnHCl to 4 M.

Enrichment of biotinylated proteins

25. Prepare High-Capacity NeutrAvidin Agarose resin for enrichment. Invert bottle several times to generate a uniform slurry, then aliquot 500 µl slurry into a 1.5-ml low-binding tube for each sample.

26. Centrifuge resin 2 min at 500 × *g*, room temperature, in a microcentrifuge. Discard supernatant.

27. Resuspend resin in 4 M GdnHCl

28. Repeat steps 26 and 27 two times.

29. Centrifuge resin 2 min at 500 × *g*, room temperature. Discard supernatant and add dissolved pellet from step 24. Incubate for at least 1 hr at room temperature on a rotator or mixer or overnight at 4°C.

    This step is a potential pause point.

30. Centrifuge resin 2 min at 500 × *g*, room temperature. Discard supernatant.

31. Resuspend resin in 500 µl of 4 M GdnHCl, and transfer to a 1-ml snap-cap spin column. Attach column to a vacuum manifold.

    If a vacuum manifold is not available, the following steps may be performed by centrifuging the column for 2 min at 500 × g, room temperature, after each wash.

32. Wash resin 10 times with 800 µl of 4 M GdnHCl.

33. Wash resin 10 times with 800 µl of 100 mM ammonium bicarbonate.

On-bead trypsin digestion

34. Remove column from the vacuum manifold, and cap the bottom. Resuspend resin in 1 ml of 100 mM ammonium bicarbonate, and transfer to a 1.5-ml low-binding tube.

35. Add 20 µg MS-grade trypsin (one tube). Incubate overnight (12 to 16 hr) on a rotating mixer at room temperature.

    Take care to avoid keratin contamination by wearing clean gloves while performing this and subsequent steps.

36. Centrifuge resin 2 min at 500 × *g*, room temperature. Discard supernatant.

37. Resuspend resin in 800 μl of 4 M GdnHCl, and transfer to a snap-cap spin column. Place on vacuum manifold.

38. Wash resin 10 times with 800 μl of 4 M GdnHCl.

39. Wash resin 10 times with 800 μl of 100 mM ammonium bicarbonate.

TEV protease elution of N-terminal peptides

40. Remove column from the vacuum manifold, and cap the bottom. Resuspend resin in 500 μl of 100 mM ammonium bicarbonate, and transfer to a 1.5-ml low-binding tube.

41. Add 2 μl of 1 M DTT.

    DTT is required for robust TEV protease activity.

42. Add 10 μg TEV protease. Incubate on a rotating mixer for 6 hr or up to overnight.

    TEV protease may be purified in-house or purchased from commercial sources.

43. Centrifuge resin 2 min at 500 × *g*, room temperature. Resuspend resin in the supernatant, and transfer to a clean snap-cap spin column. Place column in a 1.5-ml low-binding tube.

    Do not discard supernatant at this step. Centrifugation is used to spin any liquid that may have accumulated on the cap during incubation back into the tube.

44. Centrifuge column 2 min at 500 × *g*, room temperature. Save supernatant, which contains the eluted N-terminal peptides.

45. Wash resin once with 250 μl of 100 mM ammonium bicarbonate. Combine wash with the flow-through from step 44.

    This solution contains the eluted N-terminal peptides.

46. Dry sample in a vacuum concentrator.

47. Resuspend pellet in 50 to 100 μl of 5% TFA to precipitate the TEV protease. Incubate on ice for 10 min.

    Add 1 μl solution to a strip of pH paper to ensure that the pH is <3.

48. Centrifuge using a benchtop microcentrifuge 10 min at 21,000 × *g*, 4°C, to pellet precipitated TEV protease.

    The pellet from precipitated TEV protease may not be visible after centrifugation. Note the orientation of the tube in the centrifuge to avoid disrupting the pellet in subsequent steps.

Sample desalting and LC-MS/MS analysis

49. Transfer supernatant to a new tube. Desalt sample using a C18 spin desalting tip or similar device.

50. Dry desalted peptides in a vacuum concentrator.

51. Resuspend dried peptides in 10 μl of 0.1% formic acid with 2% acetonitrile for MS analysis.

52. Analyze peptides by LC-MS/MS. Use 0.1% formic acid for mobile phase A and 0.1% formic acid/80% acetonitrile for mobile phase B. Load 5 μl sample over 15 min at 0.5 μl/min in mobile phase A, and elute peptides at 0.3 μl/min with a linear gradient from mobile phase A to 40% mobile phase B over 125 min. Perform

data-dependent acquisition of MS data using Thermo Xcalibur software, scanning a mass range from 300 to 1500 *m/z*.

A typical analysis is performed on an Acclaim PepMap RSLC column using a Thermo Dionex UltiMate 3000 RSLCnano LC system coupled to a Thermo Q-Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer.

53. Generate peak lists from the RAW files using MSConvert (see Chambers et al., 2012).

54. Analyze peak lists using Protein Prospector software (see Chalkley, Baker, Medzihradszky, Lynn, & Burlingame, 2008). Search data using the human SwissProt database concatenated with randomized sequences for analysis of the false discovery rate. For data collected on a QExactive Plus, set parent ion tolerance to 6 ppm and fragment ion tolerance to 20 ppm. Choose tryptic specificity for the search, allowing nonspecific cleavages at N termini. Select carbamidomethylation at Cys as a constant modification. Select Abu at peptide N termini, acetylation at protein N termini, oxidation at Met, pyroglutamate formation at N-terminal Gln, and Met excision at protein N termini as variable modifications (Mahrus et al., 2008; Weeks & Wells, 2018).

Other search engines than Protein Prospector may be used for identification of semitryptic peptides; however, performance in semitryptic searches may vary by search engine.

REAGENTS AND SOLUTIONS

Bacillus minimal salts, 10×

To 1 L water add:
140 g K_2HPO_4
60 g KH_2PO_4
20 g $(NH_4)_2SO_4$
10 g $Na_3Citrate•2H_2O$
Sterilize by autoclaving
Store at room temperature for up to 6 months

LB agar plates

Add 40 g premixed LB medium with agar (e.g., Sigma-Aldrich, cat. no. L3147) to a 1-L autoclave-safe bottle. Bring to 1 L with water. Autoclave for 15 to 30 min at 121°C to sterilize. Cool to 55°C.
For LB agar plates without antibiotics, using sterile technique pour enough LB agar into sterile, disposable petri dishes to cover the bottom. For chloramphenicol-supplemented plates used in *B. subtilis* selection, add 1 ml of 5 mg/ml chloramphenicol dissolved in ethanol to 1 L LB agar. Mix well and pour plates. For carbenicillin-supplemented plates used in *E. coli* selection, add 1 ml of 50 mg/ml carbenicillin in water to 1 L LB agar. Mix well and pour plates. Store plates at 4°C for up to 6 months.

Lysis buffer, 4×

4% (w/v) SDS
400 mM tricine, pH 8.0
Store at room temperature for up to 1 year

Medium A

5 ml 10× *Bacillus* minimal salts (see recipe)
0.5 ml 50% (w/v) glucose
0.5 ml 1 M $MgCl_2$

0.5 ml 5 mg/ml tryptophan
0.5 ml 2% (w/v) amicase
43.3 ml water
Prepare fresh before use

The 5 mg/ml tryptophan solution should be made fresh on the day of use. Before combining, glucose, $MgCl_2$, and tryptophan solutions should be filter sterilized, and amicase and water should be autoclaved.

Medium B

1 ml 10× *Bacillus* minimal salts (see recipe)
0.1 ml 50% (w/v) glucose
50 µl 1M $MgCl_2$
5 µl 5 mg/ml tryptophan
25 µl 2% (w/v) amicase
8.82 ml water
Prepare fresh before use

The 5 mg/ml tryptophan solution should be made fresh on the day of use. Before combining, glucose, $MgCl_2$, and tryptophan solutions should be filter sterilized, and amicase and water should be autoclaved.

Ni-NTA elution buffer

50 mM sodium phosphate, pH 8.0
300 mM NaCl
250 mM imidazole
Store at room temperature for up to 1 year

The buffer can be prepared at pH ~8.0 by mixing the appropriate ratio of dibasic sodium phosphate and monobasic sodium phosphate according to the Henderson-Hasselbach equation. For example, 50 mM sodium phosphate, pH 8.0, can be prepared by mixing 12.4 g/L dibasic sodium phosphate heptahydrate and 0.47 g/L monobasic sodium phosphate monohydrate. Check the pH with a pH meter, and adjust as necessary using 6 N HCl or 10 N NaOH.

Ni-NTA wash buffer

50 mM sodium phosphate, pH 8.0
300 mM NaCl
20 mM imidazole
Store at room temperature for up to 1 year

The buffer can be prepared at pH ~8.0 by mixing the appropriate ratio of dibasic sodium phosphate and monobasic sodium phosphate according to the Henderson-Hasselbach equation. For example, 50 mM sodium phosphate, pH 8.0, can be prepared by mixing 12.4 g/L dibasic sodium phosphate heptahydrate and 0.47 g/L monobasic sodium phosphate monohydrate. Check the pH with a pH meter, and adjust as necessary using 6 N HCl or 10 N NaOH.

Subtiligase storage buffer

100 mM tricine, pH 8.0
5 mM DTT
10% (v/v) glycerol
Adjust pH to 8.0 with 10 N NaOh
Store at 4°C for up to 1 week

COMMENTARY

Background Information

Subtiligase was engineered from the broad-specificity serine protease subtilisin BPN′ from *B. amyloliquefaciens* (Abrahmsen et al., 1991). While peptide bond hydrolysis is the major reaction catalyzed by subtilisin, subtiligase has two mutations that enable it to catalyze peptide bond formation. The first is a serine-to-cysteine mutation (S221C) that reduces amidase activity while maintaining the esterase activity observed in wild-type subtilisin (Nakatsuka et al., 1987; Neet & Koshland, 1966; Polgar & Bender, 1966). The second is a proline-to-alanine mutation (P225A) that better spatially accommodates the larger cysteine nucleophile and increases peptide ligation activity by two orders of magnitude (Abrahmsen et al., 1991). Together, these two mutations minimize peptide bond hydrolysis activity but support a new peptide bond formation activity. Based on its ability to catalyze this reaction, subtiligase has been applied in site-specific, N-terminal protein bioconjugation (Chang et al., 1994; Weeks & Wells, 2018) and in cellular N terminomics (Agard et al., 2010, 2012; Julien et al., 2016; Mahrus et al., 2008; Shimbo et al., 2012), among many other applications (Jackson et al., 1994; Jackson, Burnier, & Wells, 1995).

To expand the utility of subtiligase, a new toolbox of subtiligase specificity mutants was recently developed to broaden the scope of N-terminal sequences that can be modified with subtiligase (Weeks & Wells, 2018). Engineering these mutants relied on an assay to comprehensively and quantitatively define the specificity of subtiligase and its variants. This assay, proteomic identification of ligation sites (PILS), employs proteome-derived peptide libraries as diverse pools of substrates for subtiligase. By sequencing the members of the library that are modified by subtiligase using LC-MS/MS, it was possible to determine which N-terminal sequences are the best and worst substrates for subtiligase. The PILS assay was used as a platform for engineering new subtiligase variants that are capable of efficiently modifying N-terminal sequences that are poor substrates for wild-type subtiligase, expanding the utility of the subtiligase method. Further, PILS data collected for each mutant can be used to determine which mutant is the best choice for executing the protocols described here for any given target protein(s). These data are available in a searchable format through the ALPINE web application (*https://wellslab.ucsf.edu/alpine*).

Critical Parameters

Reaction pH

Reaction pH is an important consideration in both Basic Protocol 1 and Basic Protocol 2. The reaction pH must be >7 to support efficient catalysis by subtiligase based on its catalytic triad and reaction mechanism. The optimal pH for modification with subtiligase is 8 to 8.5.

Target protein sequence and structure

The target protein sequence and structure are key considerations for the success of a subtiligase modification reaction. For Basic Protocol 1, the N-terminal sequence of the protein to be modified should be matched with the subtiligase variant that modifies that sequence efficiently. For most sequences, multiple subtiligase variants exist that catalyze efficient modification. All subtiligase variants that will modify a particular sequence can be identified using the ALPINE web application. N-terminal secondary structure and accessibility are also key factors in the success of a subtiligase reaction. As described in the Strategic Planning section, if a particular protein of interest has unfavorable N-terminal structure or accessibility, adding an N-terminal extension or supplementing the reaction mixture with detergents or chaotropic agents may improve the chances of success.

Avoidance of interfering protease activity

Because the subtiligase substrate is a labile ester, it may be rapidly cleaved by proteases in cell lysate or contaminants in purified proteins, rendering it unreactive for acylating the catalytic cysteine of subtiligase. Protease inhibitors should be used during purification of target proteins to be used in Basic Protocol 1 when possible. Basic Protocol 2 also describes the use of specific protease inhibitors that will inhibit cellular proteases but not subtiligase.

Troubleshooting

Table 1 lists common problems encountered during the execution of Basic Protocols 1 and 2 and possible solutions to overcome these problems.

Understanding Results

Anticipated results for Basic Protocol 1 are shown in Figure 2. For a target protein that

Table 1 Troubleshooting Guide for Subtiligase-Catalyzed Protein Bioconjugation and Subtiligase N Terminomics

Problem	Possible cause	Possible solution
Low bioconjugation efficiency	Target protein considerations	See Strategic Planning section
	Reaction pH	Test reaction pH and adjust to pH 8
Low modification efficiency in cell lysate	Reaction pH	Test reaction pH and adjust to pH 8
	Active proteases in the sample are hydrolyzing the peptide ester	Add protease inhibitors
	DTT was not added to quench iodoacetamide	Add DTT to 25 mM final concentration
No Abu-tagged peptides detected by LC-MS/MS	Many possible causes	Follow biotinylated proteins by streptavidin blot to detect where they are lost
		Check search parameters used to identify peptides from the peak list file

Abu, aminobutyric acid; DTT, dithiothreitol; LC-MS/MS, liquid chromatography tandem mass spectroscopy.

is a good substrate for the chosen subtiligase variant, near-quantitative modification can be expected. For substrates with an N terminus that is somewhat inaccessible, more than one round of subtiligase labeling may be required to achieve near-complete modification. For target proteins that are poor substrates for the chosen subtiligase variant because their sequences are incompatible, little to no modification is observed. However, modification is improved when a compatible subtiligase variant is chosen. For target proteins that are poor subtiligase substrates because their N termini are inaccessible, little to no modification is expected. However, modification efficiency can be improved by extending the N terminus.

Anticipated results for Basic Protocol 2 are shown in Figure 7. Hundreds of N-terminal semitryptic peptides are expected to be identified in both treated and untreated samples. For untreated cell lysate, very few N termini following an aspartate residue in the protein sequence are expected. For an etoposide-treated cell lysate, a large number of N termini that follow an aspartate are expected.

Time Considerations

Execution of Basic Protocol 1 requires 4 hr or less. However, purification of subtiligase (Support Protocol 1) and synthesis of the subtiligase substrate (Support Protocol 2) each require ∼2 days. Each support protocol is expected to yield enough reagent to last for many bioconjugation experiments.

Basic Protocol 2 can be completed in 4.5 days after cells are ready for etoposide treatment. One day is required for etoposide treatment, and 2.5 days are required for subtiligase labeling and enrichment of N-terminal peptides. After N-terminal peptides are isolated, ∼4 hr are required to analyze each sample by LC-MS/MS.

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Internet Resources

https://wellslab.ucsf.edu/alpine

The ALPINE (α-Amine Ligation Profiling Informing N-terminal modification Enzyme selection) web application enables algorithmic selection of the most efficient subtiligase variant(s) for modification of a particular N-terminal sequence or group of sequences.