Expressed Protein Ligation using subtiligase

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Method Article

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Abstract

Expressed protein ligation is a valuable method for protein semisynthesis that involves the reaction of recombinant protein C-terminal thioesters with N-Cys containing peptides but the requirement of a Cys residue at the ligation junction can limit its use. Here we present a method that employs subtiligase to efficiently ligate Cys-free peptides to protein thioesters. 

Introduction

The ability to site-specifically install post-translational modifications, biophysical probes, unnatural amino acids, isotopic labels, and drug-like small molecules into proteins of any size offers enormous potential for both fundamental and applied biomedical research. Semisynthesis using expressed protein ligation \(EPL) has been exploited frequently in the construction of proteins containing diverse chemical modifications. In standard EPL, a recombinant protein fragment is fused to an intein, which is then reacted with a thiol to generate the isolated recombinant protein C-terminal thioester. An N-Cys containing synthetic peptide is then added to the protein thioester which undergoes transthioesterifcation followed by rearrangement to a conventional amide bond. While powerful, the scope of EPL is narrowed by the requirement of a Cys at the ligation junction. Subtiligase is an engineered peptide ligase derived from the protease subtilisin that contains mutations \(S221C, P225A) which alter the mechanism to favor aminolysis over peptidase activity, facilitating the ligation of a peptide containing a C-terminal ester or thioester to a peptide containing an α-amine. We describe here a general method for generating a protein thioester from a protein-intein fusion, and using subtiligase to ligate it to a synthetic peptide.

Reagents

E. coli DH5α for cloning _E. coli_ BL21 for expression SF21 and High Five insect cells for expression Lysis buffer: 250 mM NaCl, 50 mM HEPES, 1 mM EDTA, 10% glycerol, 0.1% Triton X-100, pH = 7.5 Wash buffer: 250 mM NaCl, 25 mM HEPES, 0.1% Triton X-100, pH = 7.5 Cleavage buffer: 250 mM NaCl, 50 mM HEPES, 1 mM EDTA, pH = 7.5 Storage buffer: 150 mM NaCl, 50 mM MES, pH = 6-6.5. The thioester is more stable at a lower pH, but pH up to 6.5 is fine if the protein of interest cannot tolerate a lower pH. Reaction buffer: 10 mM $CaCl_2$, 100 mM BICINE, pH = 8.0 Chitin Resin Sodium Mercaptoethane sulfonate \(MESNA) Subtiligase \(100 μM stock)

Equipment

Equipment for protein expression culture Equipment for cell lysis, e.g. French press cell for _E. coli_ or homogenizer for insect cells. Floor and table centrifuges Empty Biorad gravity flow column Heat block capable of maintaining 25°C SDS-PAGE system

Procedure

1. Subclone the cDNA for a protein fragment of interest N-terminal to an intein-CBD-containing vector \(available from New England BioLabs). In the first and fourth residues upstream of the intein, in the protein fragment, avoid acidic residues and where possible include hydrophobic residues. 2. Express the fusion protein in the appropriate heterologous host \(E. coli or baculovirus-SF9/High Five insect cells). 3. Lyse the cells by an appropriate method. 4. Clear the lysate by centrifuging at 15,0000 rpm for 45 min. at 4° C. If insect cells were used, incubate the cleared lysate with powdered cellulose to remove chitinase \(about 1 ml cellulose for every 2 ml lysate). 5. In a cold room, immobilize the fusion protein on chitin resin and wash to remove impurities. 6. Treat the immobilized fusion protein with cleavage buffer containing 300 mM MESNA overnight at room temperature and isolate the protein thioester in the eluate. 7. Exchange the protein thioester \(by dialysis or ultrafiltration, as needed) into storage buffer. 8. Concentrate the protein to 1 mg/mL or greater. If not used within a day, flash-freeze for storage. 9. Mix the protein thioester \(0.4 mg/mL or greater) with synthetic peptide \(1-10 mM) and subtiligase \(0.5-25 M) in reaction at room temperature. 10. After 5-90 minutes, analyze the reaction mixture using SDS-PAGE to determine degree of ligation. 11. Once the ligation is optimized, purify the ligated protein using chromatography appropriate for the protein of interest.

Timing

Creating protein thioester: 24 hours \(4 - 6 hours + overnight incubation) Ligation: 2 - 4 hours

Troubleshooting

If ligation efficiency is low: reposition the ligation site so that hydrophobic residues are at the 1st and 4th positions upstream of the ligation site. Avoid acidic residues in these positions, or at the C-terminus of the peptide. Use greater concentrations of peptide and/or subtiligase. For peptide/proteins with acidic residues at the ligation junction use E156Q,G166K or Y217K variants of subtiligase. For peptides containing phosphates, use a high-calcium buffer \(up to 100 mM). In all cases, longer reaction times may be necessary.

Anticipated Results

After analyzing the reaction by SDS-PAGE, there should be three main bands in the reaction lanes: subtiligase \(around 27 kDa), the ligation product, and below it, unreacted protein thioester hydrolyzed by subtilgase. See "Figure 2":http://www.nature.com/protocolexchange/system/uploads/4737/original/EnzymeResult.jpg?1472494297 for an example ligation between PTEN and a 25-mer peptide.

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Figures

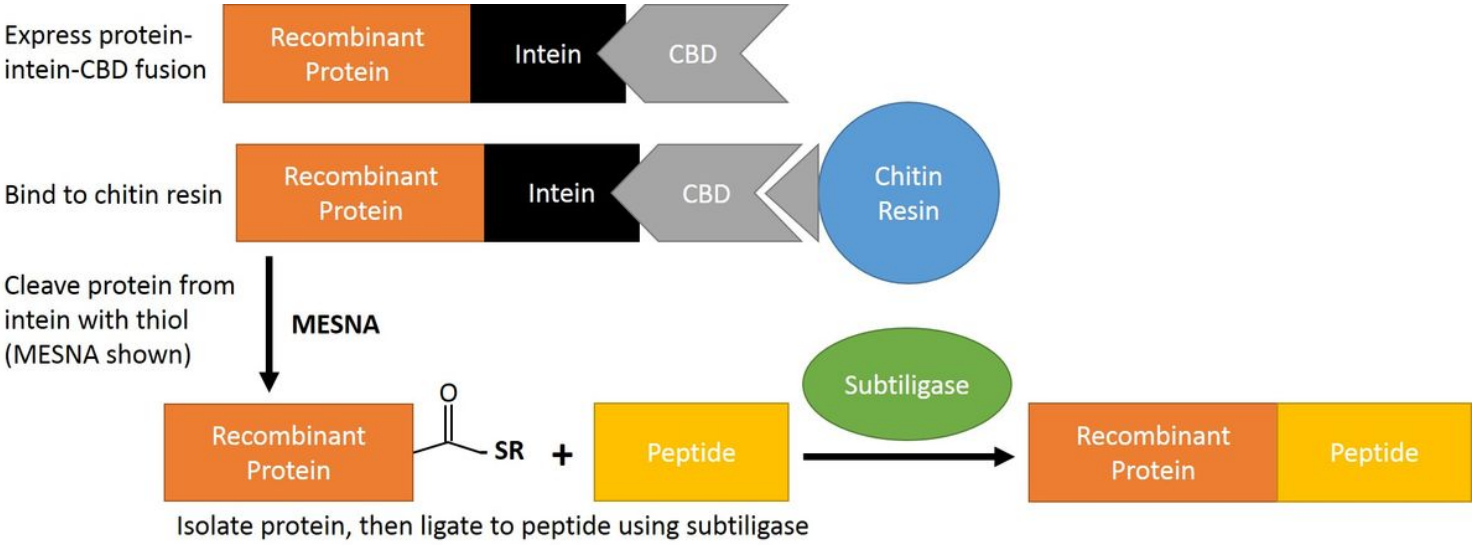

Figure 1

Schematic for subtiligase ligation schematic illustrating the generation of a protein thioester by reaction the protein-intein fusion with a thiol (MESNA shown here), then ligating that protein thioester to a peptide using subtiligase

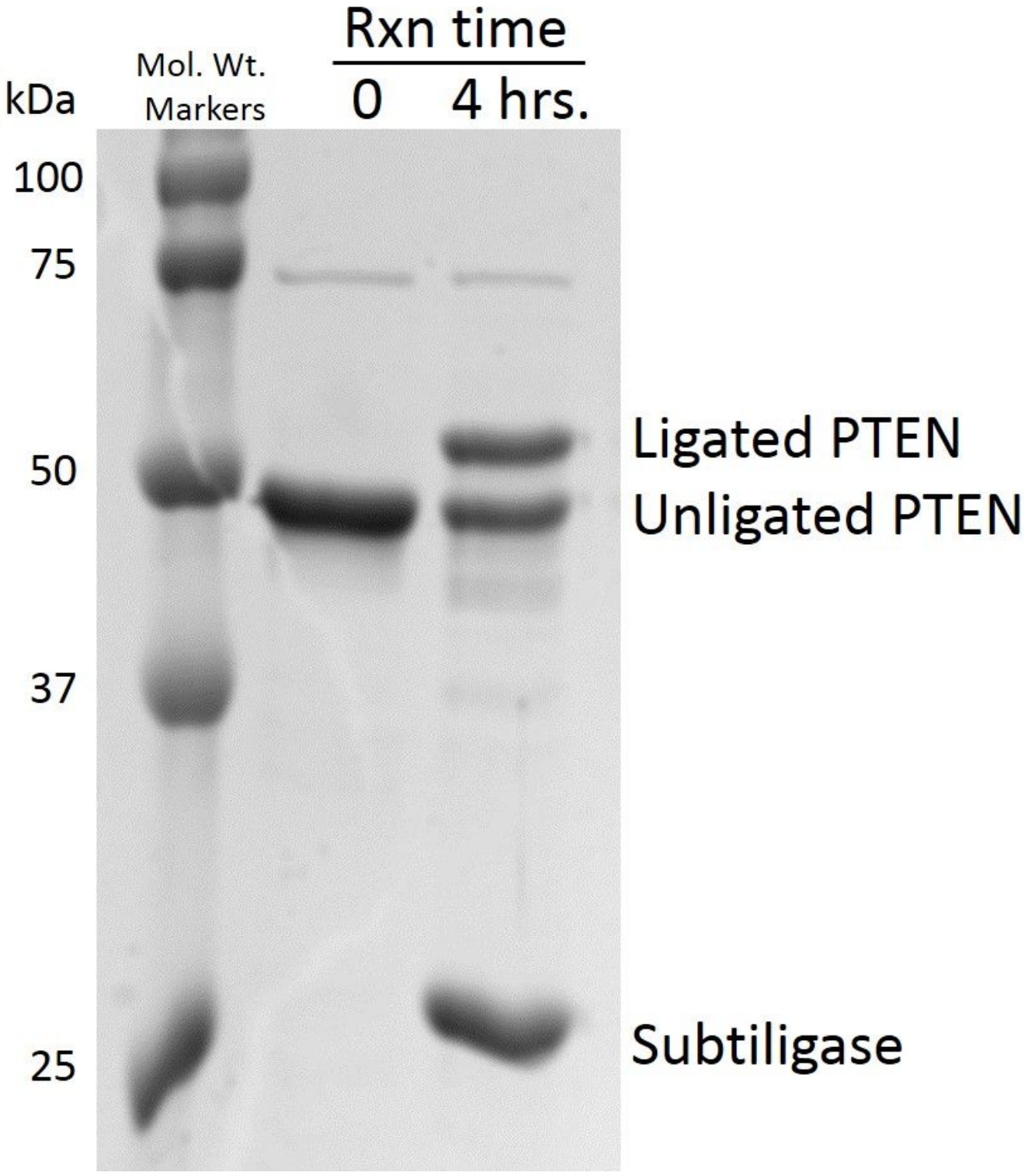

Figure 2

Result of ligation between PTEN and 25-mer peptide SDS-PAGE analysis of the result of a 4-hour ligation between r-PTEN and non-phosphorylated tail peptide. Ligation proceeded to ~50% completion