Butelase-mediated synthesis of protein thioesters and its application for tandem chemoenzymatic ligation†

Yuan Cao, Giang K. T. Nguyen, James P. Tam* and Chuan-Fa Liu*

Using a recently discovered peptide ligase, butelase 1, we developed a novel method to access protein thioesters in good yield. We successfully combined it with native chemical ligation and sortase-mediated ligation in tandem for protein C-terminal labeling and dual-terminal labeling to exploit the orthogonality of these three ligation methods.

Over the past three decades, considerable efforts have been devoted to developing chemoselective peptide ligation strategies that operate under aqueous conditions and without the use of a protection group scheme to enable protein synthesis and site-specific tagging of macromolecules for various biochemical applications. The chemical strategies thus developed often exploit the unique bifunctional moiety of a peptide’s N-terminal amino-acid residue that has a nucleophilic side chain such as Cys or Ser/Thr to react with another peptide containing a C-terminal “activated” moiety. These include, in chronological order, pseudo-proline ligation, native chemical ligation, thioacid capture ligation, and serine/threonine ligation, although the last one requires a non-aqueous solvent system. The most successful of these is native chemical ligation (NCL) to which the key is the use of a peptide C-ter thioester of aryl or alkyl nature for mediating the ligation reaction with a cysteinyl moiety. Besides being used in NCL, peptide thioesters are also used as building blocks in the more classical peptide segment condensation method via silver ion activation. Thus, an efficient and convenient method to prepare a peptide/protein thioester is always desirable and continues to be well sought after. Whereas peptide thioesters can be synthesized directly by Boc chemistry or indirectly by Fmoc chemistry, protein thioesters are obtained mostly through intein technology. This method requires the fusion expression of the target protein with an engineered intein and the addition of an external thiol to release the protein thioester from the immobilized intein (Fig. 1A). Other methods using subtiligase or SrtA to prepare peptide/protein thioesters have also been developed.

Recently a novel Asn/Asp(Asx)-peptide ligase, butelase 1, was discovered. Butelase 1 performs a cyclization step in the cyclotide biosynthesis in the medicinal plant Clitoria ternatea. It recognizes an −N(D)HV motif at the C-terminus and cleaves the N(D)–H bond to conjugate with an incoming N-terminal amino-acid residue (Xaa) to form an Asx-Xaa at the ligation junction. Butelase 1 has been shown to achieve success in both inter- and intramolecular peptide ligation with a high catalytic efficiency and nearly “traceless” features.

Herein we report a butelase-mediated ligation (BML) method for the preparation of protein thioesters (Fig. 1B) with high efficiency. We further coupled this method with NCL and sortase-mediated ligation (SML) for labelling proteins to show that these three ligation strategies are orthogonal to each other.

Four glycine thioesters Gly-COSR (Table 1) were first used to demonstrate butelase-mediated thioester synthesis. Since these simple glycyl thioesters were easily synthesized from...
Boc-Gly–OH and commercially available thiols, one could afford to use them in large excess (44 fold to the acyl peptide substrate, Table 1) to outcompete the reverse reaction, essentially overcoming the reversibility problem of BML. As a result, all four glycine thioesters ligated efficiently with the model peptide YKNHV in the presence of butelase 1 to afford the corresponding peptide thioesters YKNG-COSR \(3a-d\) in near quantitative yields (Table 1 and Fig. S1, ESI†) as analysed by HPLC. To determine the pH effect of this enzymatic ligation, reaction between \(1b\) and \(2\) was performed under different pH conditions: pH 4, 5, 6, 6.5, 7.5 and 8. We found that the reaction proceeded efficiently at pH 5, 6 and 6.5 (> 90%), and less efficiently at pH 4, 7.5 and 8. This result indicates that butelase 1 performs the ligation optimally under mildly acidic pH conditions, a feature desirable for the stability of thioesters. For convenience, we used the glycine thioester \(1b\) in our following work. We further prepared a series of peptides \(4a-f\) with a common sequence YKNHV varying the residue at the P2 position (X = Val, Leu, Phe, Ser, Nle, D-Ala). All six peptides, including two peptides containing unnatural amino acids, ligated with excessive glycine thioester \(1b\) at pH 6 to afford the respective products YXNG-COSR \(5a-f\) in good yields after 95 min (Table 2). This result clearly showed the generality of our method and that butelase 1 tolerates a variety of amino acids N-terminal to the NHV tripeptide recognition motif. Clearly, an advantage of this approach is that a large excess of Gly-COSR can be used to force the reaction to completion in a short duration.

We also tested the thioesters of four other amino acids (Ala, Leu, Ile, Phe) in butelase-mediated ligation with model peptide 2. Surprisingly, only alanine thioester gave some modest ligation products, while the other amino-acid thioesters did not work in

### Table 1

<table>
<thead>
<tr>
<th>Glycine thioester</th>
<th>R</th>
<th>Yield [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>R1</td>
<td>&gt; 95</td>
</tr>
<tr>
<td>1b</td>
<td>R2</td>
<td>&gt; 95</td>
</tr>
<tr>
<td>1c</td>
<td>R3</td>
<td>&gt; 95</td>
</tr>
<tr>
<td>1d</td>
<td>R4</td>
<td>&gt; 95</td>
</tr>
</tbody>
</table>

All reactions were carried out under the same conditions: 50 μM peptide, 2.2 mM glycine thioester, 100 nM butelase 1, pH 6, 20 mM phosphate buffer, 42 °C. Yields were measured after 2 h when reactions reached equilibrium.

### Table 2

<table>
<thead>
<tr>
<th>Peptide</th>
<th>X</th>
<th>Yield [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>4a</td>
<td>Val</td>
<td>&gt; 95</td>
</tr>
<tr>
<td>4b</td>
<td>Leu</td>
<td>93</td>
</tr>
<tr>
<td>4c</td>
<td>Ser</td>
<td>81</td>
</tr>
<tr>
<td>4d</td>
<td>Phe</td>
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</tr>
<tr>
<td>4e</td>
<td>Nle</td>
<td>&gt; 95</td>
</tr>
<tr>
<td>4f</td>
<td>d-Ala</td>
<td>91</td>
</tr>
</tbody>
</table>

All reactions were carried out under standard conditions: 50 μM peptide, 2 mM glycine thioesters, 100 nM butelase 1, 20 mM phosphate buffer, pH 6, 42 °C. All yields were measured after 95 min when reactions reached equilibrium.

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![Fig. 2 Butelase-mediated thioester synthesis of (A) ubiquitin, masses observed in ESI-MS for the starting material \(6\) and product \(7\) are 9982.5 Da (calc. 9982.3 Da) and 9066 Da (calc. 9066.2 Da), respectively. (B) GFP, masses observed in ESI-MS for the starting material \(8\) and product \(10\) are 29724 Da (calc. 29725.7 Da) and 29631 Da (calc. 29632.6 Da), respectively. (C) DARPin(ERK), masses observed in ESI-MS for the starting material \(9\) and product \(11\) are 20215 Da (calc. 20214.4 Da) and 20122 Da (calc. 20121 Da), respectively.](image-url)
the ligation reaction (data are not shown). So, a limitation of this method is that, among the amino-acid thioesters, only glycinyl thioesters are efficient substrates of butelase 1.

To show the compatibility of BML with NCL, the peptide thioester YKNG-COSR \(3b\) was ligated with a cysteinyl peptide CGYKNHV to afford YKNCGYKNHV in quantitative yield under standard NCL conditions (Fig. S2, ESI†).

To show the utility of this approach for proteins, we prepared an engineered ubiquitin \(6\) with the NHV recognition motif and a His\(_6\)-tag for affinity purification at its C-terminus. Reaction of \(6\) (100 \(\mu\)M) with glycine thioester \(1b\) (2 mM, 20 eq.) in the presence of 200 nM butelase 1 (0.002 eq.) afforded the protein thioester product 7 in >90% yield after 2 h (Fig. 2A). We also prepared two other proteins: GFP \(8\) and an ERK-ankyrin repeat protein (DARPin(ERK))\(15\) \(9\), both engineered to have a C-ter NHV motif. Using the same reaction, we obtained the thioester products \(10\) and \(11\) in ca. 85% yield in short time (Fig. 2B and C). These results show again the high catalytic efficiency of butelase 1. Since the \(-\text{NHV}\) motif is very small, any protein tagged with it can be easily expressed as shown in the three protein examples here. These features make our approach a very robust and convenient method for the preparation of protein thioesters.

Next, we showed that the prepared protein thioester was useful for NCL. So, a cysteinyl peptide CK(biotin)LKVA \(12\) was ligated with the ubiquitin thioester 7 under standard NCL conditions to afford, in near quantitative yield as observed by HPLC analysis (Fig. 3), the product ubiquitin \(13\) containing now a biotin probe at its C-ter end.

To show that SML can also be used in conjunction with the above two reactions for a three-step tandem ligation, another recombinant ubiquitin \(14\) was prepared. It contained a \(-\text{NHV}\) motif followed by a His\(_6\)-tag at the C-terminus. At the N-terminus, it displayed a glycine residue for SrtA recognition. One of the practical applications of this convergent methodology could be the dual-terminus modification of proteins (Fig. 4A). This attempt was previously performed by using two sortases with different activities.\(^{16}\) In our case, the C-terminal labeling of \(14\) was first conducted using the above two-step process: BML with glycine thioester \(1b\) followed by NCL with biotinyl peptide \(12\). 85% and quantitative HPLC conversions were obtained for the BML and NCL steps, respectively, to give \(15\) and \(16\) (Fig. S3 and S4, ESI†). After HPLC purification, the C-terminus-labeled ubiquitin \(16\) was further labeled on the N-terminus using SrtA and a depsipeptide containing a LPET-glc-G motif. The use of the ester derivative of the SrtA motif LPXTG was based on a previous report that it could significantly improve the SML yield due to the irreversibility of the reaction.\(^{17}\) We placed a short sequence \(\text{GSGS}\) between the N-terminal Gly residue and
the main body of ubiquitin for better accessibility of its N-terminus by SML. A depsipeptide Nle-YLPET-glc-G 17a was first used for demonstration. The SML reaction gave a near quantitative yield of 18a after 2.5 h with two molar equivalents of the depsipeptide and 0.1 molar equivalent of SrtA (Fig. 4B). SML requires a high molar ratio of SrtA, which is a significant drawback as compared to BML, which requires a very small amount of the enzyme. Nevertheless, a simple treatment with Ni-NTA removed the His-tagged SrtA and subsequent dialysis removed the small peptide from the desired product. Using SML, 16 was also successfully ligated with a fluorescein-peptide fluor-YLPET-glc-G 17b to give 18b which is dually labelled with a fluorescent probe and a biotin group on the N- and C-termini, respectively (Fig. 4C).

Currently, intein-based technology is the method of choice to access protein thioesters for ligation reactions. We have shown that BML provides a complementary method to prepare protein thioesters conveniently and efficiently as seen with several protein substrates of different sizes. This method showed that BML provides a complementary method to prepare protein thioesters for ligation reactions. We have further shown that this method can be combined successfully in tandem with NCL and SML for sequential ligation to achieve bi-directional and orthogonal labelling of ubiquitin in a model system. These results demonstrate butelase 1 as a versatile tool for protein manipulation. We foresee that, like other emerging methods, BML will offer numerous future opportunities in biotechnology.

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Notes and references