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Streamlined Expressed Protein Ligation Using Split Inteins

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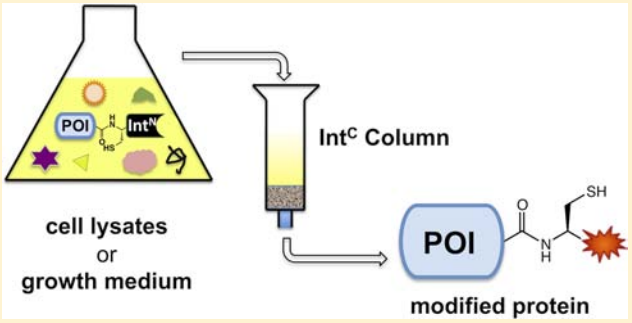
Supporting Information

ABSTRACT: Chemically modified proteins are invaluable tools for studying the molecular details of biological processes, and they also hold great potential as new therapeutic agents. Several methods have been developed for the site-specific modification of proteins, one of the most widely used being expressed protein ligation (EPL) in which a recombinant α-thioester is ligated to an N-terminal Cys-containing peptide. Despite the widespread use of EPL, the generation and isolation of the required recombinant protein α-thioesters remain challenging. We describe here a new method for the preparation and purification of recombinant protein α-thioesters using engineered versions of naturally split DnaE inteins. This family of autoprocessing enzymes is closely related to the inteins currently used for protein α-thioester generation, but they feature faster kinetics and are split into two inactive polypeptides that need to associate to become active. Taking advantage of the strong affinity between the two split intein fragments, we devised a streamlined procedure for the purification and generation of protein α-thioesters from cell lysates and applied this strategy for the semisynthesis of a variety of proteins including an acetylated histone and a site-specifically modified monoclonal antibody.

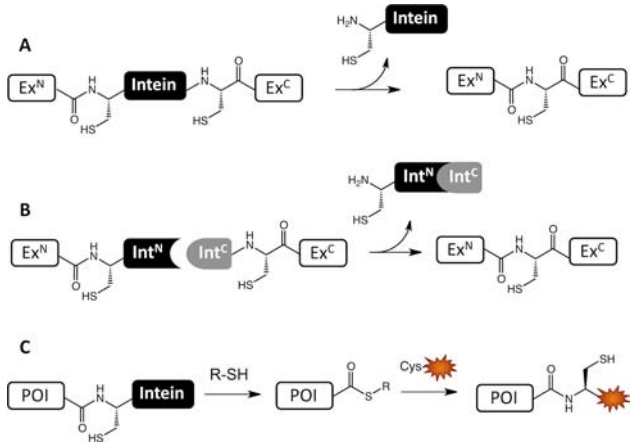


INTRODUCTION

The chemical modification of proteins is an established tool for studying the structure, function, and regulation of this class of biopolymer.[1] Moreover, in recent years, a great deal of effort has been directed toward the modification of proteins for therapeutic applications.[2] Traditionally, protein conjugation chemistries have exploited the reactivity of surface-exposed nucleophilic amino acids, such as cysteine or lysine, however, these methods typically result in heterogeneous mixtures of products, which can complicate biological studies or efficacious medicinal applications. To address this concern, several strategies have been developed for the site-specific modification of proteins, ranging from total chemical synthesis (usually via native chemical ligation, NCL)[3] to the genetic incorporation of unnatural amino acids[4] or bio-orthogonal functional groups.[5] In between these two extremes lie a variety of semisynthetic approaches.[5−7]

The most widely used protein semisynthesis technique is an extension of NCL termed expressed protein ligation (EPL) in which a recombinant protein α-thioester building block is ligated to a synthetic molecule equipped with a 1,2-aminothiol moiety (most commonly an N-terminal Cys-containing peptide) through the formation of a native peptide bond[8,9] (Scheme 1). Since its inception, EPL has been applied to a wide variety of proteins, including enzymes,[10] ion channels,[11] transcription factors,[12] transmembrane receptors,[13] and antibodies[14] (for reviews see refs 15 and 16). One of the basic requirements of EPL is a thioester group at the C-terminus of a

Scheme 1. Protein Splicing (A), trans-Splicing (B) and EPL (C)[a]



[a]Ex^N and Ex^C represent N- and C-exteins, respectively. Int^N and Int^C represent N- and C-intein fragments, respectively.

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recombinant protein. This reactive handle is introduced by exploiting a process known as protein splicing (Scheme 1), which is mediated by an autoprocessing domain called an intein.[16,17] Protein splicing typically takes place through the formation of one or more protein thioester intermediates, which ultimately resolve to form a native peptide bond between the sequences flanking the intein (referred to as N- and C-exteins). By using appropriate intein mutants, it is possible to intercept these intermediates with exogenous thiols, resulting in an N-extein of choice being cleaved from the mutant intein as a reactive α-thioester derivative suitable for chemical ligation.[8,18]

Despite the many successes of EPL, the approach often suffers from low overall efficiency due to complications associated with the generation of protein α-thioesters. In particular, fusions to inteins are, to varying extents, susceptible to premature extein cleavage, both *in vivo* and during initial purification from cell lysates, which reduces the isolated yield of the intein fusion needed for the subsequent thiolysis step.[19] Importantly, the cleaved extein side-product is unreactive toward EPL, and its separation from the desired α-thioester or the ligation product is often difficult for large proteins, such as antibodies.[19] To compound matters, the thiolysis reaction itself can be slow and inefficient, further strengthening the need to develop customized purification regimes, involving multiple chromatographic steps, to isolate the desired product from complex mixtures.[20−22] Collectively, these technical issues mean that a considerable investment in time and resources is usually required before a semisynthetic protein is obtained in useful quantities.

To overcome the various drawbacks associated with the intein thiolysis process central to EPL, we envisioned an alternative strategy based on naturally occurring split inteins. Unlike inteins used in standard EPL, which are contiguous polypeptides that catalyze protein splicing in *cis*, split inteins consist of two discrete polypeptides, herein termed Int^N and Int^C, which, upon association, catalyze protein splicing in *trans* (protein *trans*-splicing, PTS, Scheme 1).[23] Split inteins have two important properties that make them attractive for an improved EPL strategy. First, cognate Int^N and Int^C pairs often bind tightly to one another; dissociation constants in the low nanomolar range, reflecting extremely fast on-rates, have been reported for members of the naturally split DnaE inteins from cyanobacteria.[24,25] This ability of split inteins to self-associate has recently been exploited by Lu et al. as part of a traceless protein purification system, in this case using an artificially split intein pair.[26] The potential utility of split inteins in EPL is further enhanced by the remarkable splicing efficiency of some members of the family.[27,28] For instance, many of the split DnaE inteins have half-lives for the splicing reaction of less than a minute, as compared to several hours in the case of the *cis*-splicing inteins commonly used in EPL.[27,28] Moreover, recent mechanistic investigations indicate that these "ultrafast" DnaE inteins have a highly activated N-terminal splice junction, making them superior reagents for protein α-thioester generation.[28] Given the unique properties of split inteins, in particular, the ultrafast split DnaE inteins, we conceived the integrated protein modification system shown in Figure 1 in which the split intein association is employed both to purify the desired protein from complex biological mixtures and to trigger the generation of a desired protein α-thioester for EPL. In principle, this complementation system should address the major issues attendant to the standard EPL protocol, including premature cleavage of the intein, which cannot occur in the case of a split intein fragment absent its cognate partner.[29,30]

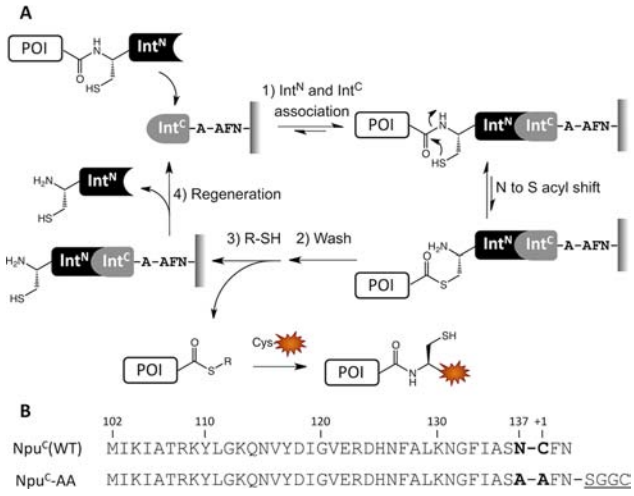

Figure 1. Streamlined EPL using split inteins. (A) Schematic of the procedure for the isolation of an α-thioester derivative of a protein of interest (POI) using engineered split intein fragments (Int^N and Int^C). EPL can be performed in a one-pot fashion during thiolysis from the split intein or immediately after elution, without need of any further purification. (B) Sequences of Npu^C (WT) and the Npu^CAA mutant used on the split intein column. Catalytic residues mutated in Npu^CAA are shown in bold, and the linker sequence added for immobilization onto the solid support is underlined. Sequences are numbered according to the intein sequence alignment shown in Figure S2.

RESULTS AND DISCUSSION

Split Intein Mediated Thiolysis and EPL. To implement our system, we designed a mutant of the ultrafast *Nostoc punctiforme* (Npu) split DnaE intein suitable for efficient α-thioester generation. Specifically, we mutated the catalytic C-terminal residue in the Int^C fragment (Asn137) and the first residue in the C-extein (Cys+1) to Ala, to allow for efficient build up of the desired splicing intermediates upon exposure to an N-extein-Npu^N fusion (Figure 1). Preliminary studies showed that mixing N-extein-Npu^N fusions (where N-extein corresponded to various model proteins) with the mutant Npu^C (Npu^CAA) led to highly efficient N-extein α-thioester formation in a thiol-dependent manner (Figures S3 and S4). Importantly, only very low levels of intein cleavage (i.e., unwanted hydrolysis) were observed in the absence of thiols, thereby fulfilling a requirement of our integrated strategy.

Encouraged by these results, we adapted the system for the one-pot purification and generation of C-terminally modified proteins by taking full advantage of the strong and specific interaction between the split intein fragments. Accordingly, the Npu^CAA peptide was immobilized on a solid support through a unique Cys residue engineered within its C-extein region (Figure S5). The resulting Npu^CAA column (hereafter referred to as Int^C column) was then evaluated as an affinity-modification resin. Three test proteins maltose binding protein (MBP), ubiquitin (Ub), and protein histidine phosphatase type 1 (PHPT1) were genetically fused to Npu^N and expressed in *Escherichia coli*. In each case, cells were lysed, and the soluble fraction was loaded onto the Int^C column to allow binding of the Npu^N tagged protein to the immobilized Npu^CAA. After a brief incubation (~ 5 min at rt) the column was extensively washed to remove contaminants, and thiolysis was triggered by

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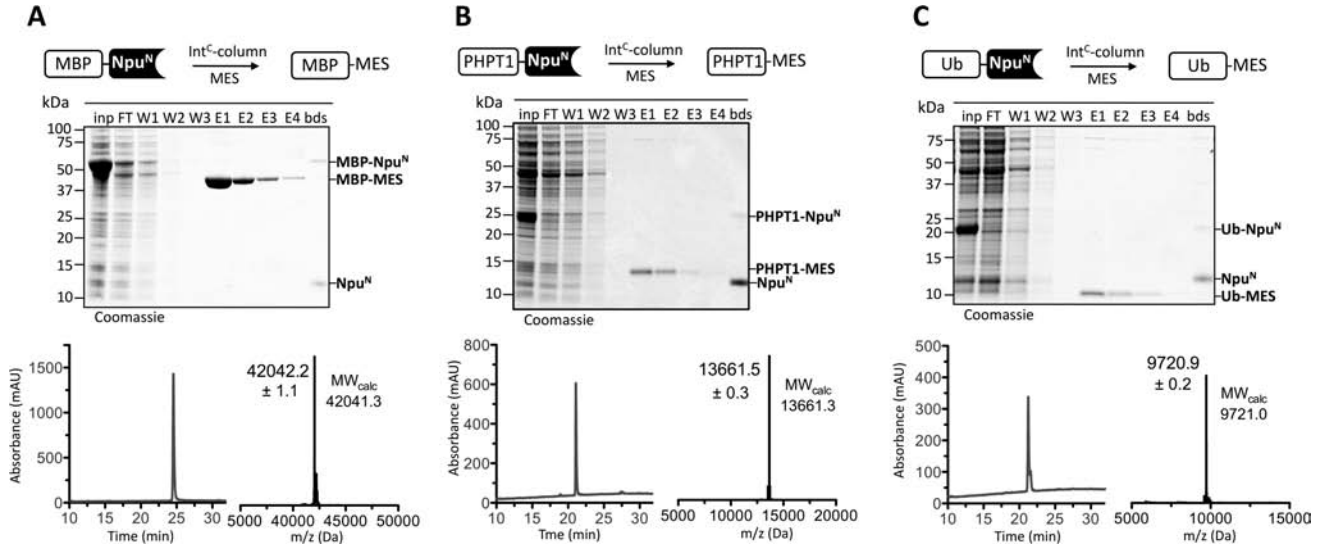

Figure 2. Purification of α-thioester proteins expressed in *E. coli*. (A) MBP, (B) PHPT1, and (C) Ub mercaptoethansulfonate (MES) α-thioesters were purified in one step from *E. coli* cell lysates using the Int^C column. The purifications were monitored by coomassie stained SDS-PAGE analysis (top) (inp: input, FT: column flow-through, W1-3: washes, E1-4: elutions, and bds: resin beads). RP-HPLC (detection at 214 nm) and ESI-TOF MS analysis of the eluted fractions (bottom left and right, respectively) confirmed the identity of all protein α-thioesters and indicated high purity.

addition of a buffer containing the thiol, mercaptoethansulfonate (MES). In all three cases, the desired α-thioester protein eluted from the Int^C column with high recovery (75−95%) and high purity (~95% as determined by RP-HPLC and mass spectrometry) (Figure 2). Total isolated yields of purified protein α-thioesters varied from one protein to another and ranged from 2.5 mg (per L of bacterial culture) for Ub-MES to 40 mg for MBP-MES. The calculated loading capacity of the Int^C column used in these experiments was 3−6 mg of protein per mL of beads (0.12 μmols/mL), but higher or lower loadings could easily be achieved by modifying the amount of Npu^C-AA immobilized on the solid support. Furthermore, we showed that the Int^C column could be regenerated and reused at least 5 times with only minimal loss of capacity (Figures S11 and S12). The utility of the α-thioester derivatives of Ub, MBP, and PHPT1 obtained from the column was demonstrated by ligating each of them to an N-terminal Cys-containing fluorescent peptide (CGK(Fl)) to give the corresponding semisynthetic products in excellent yield (Figure S10). Importantly, one-pot thiolysis/ligation reactions could be carried out, which allowed us to obtain a site-specifically modified protein directly from cell lysates without isolating the intermediate thioester (Figure 3).

Dependance on the Identity of the C-Terminal Amino Acid. An attractive feature of EPL is that it can allow for the preparation of site-specifically modified proteins in a virtually traceless manner. This is contingent on the ability to efficiently generate recombinant protein α-thioesters when any of the 20 proteinogenic amino acids are present at the C-terminus of the protein. The activity of split inteins is known to be sensitive to the identity of the amino acids immediately flanking the splice junction.[28,31,32] Thus, we were eager to test the generality of our strategy and asked whether we could generate α-thioesters of all 20 amino acids, using ubiquitin as the N-extein template. Twenty Ub-Npu^N fusion proteins were individually expressed in *E. coli* and purified over the Int^C column as before. Thiol-induced cleavage yields from the solid support were determined by SDS-PAGE analysis of the eluted and resin beads fractions, and levels of competing side reactions (mainly hydrolysis) were

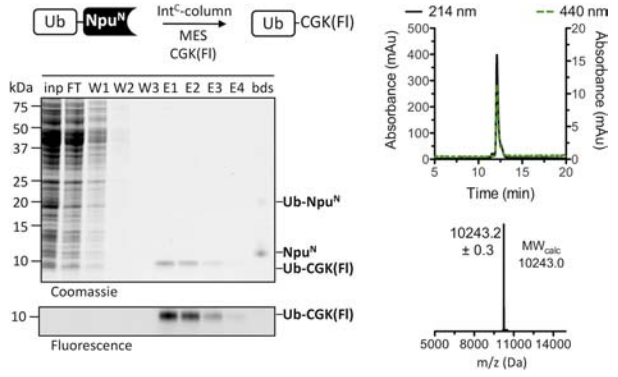

Figure 3. One-pot purification/ligation of ubiquitin to the H-CGK(Fluorescein)-NH$_2$ peptide (CGK(Fl)). Ub-Npu^N from *E. coli* cell lysates was bound to the Int^C column, as shown in Figure 2, and after removal of contaminants through extensive washes, intein cleavage and ligation were triggered by addition of 200 mM MES and 1 mM CGK(Fl) peptide. Coomassie stained SDS-PAGE analysis and in gel fluorescence of the purification/ligation (left). RP-HPLC (detection at 214 and 440 nm) and ESI-TOF MS (right) of the eluted fractions confirm the desired ligated protein was obtained in one step directly from cell lysates with a ligation yield close to 95% (quantified by RP-HPLC).

measured by RP-HPLC and ESI-TOF MS. The results clearly show that for most amino acids >60% of the bound proteins were recovered after MES treatment. Furthermore, 80−95% of the eluted material was the desired α-thioester product. The only exceptions were Pro and Glu, for which recovery were 49 and 50%, respectively (Figure 4). The Asn mutant displayed high levels of cleavage from the split intein, but almost no α-thioester could be isolated due to side-chain cyclization to form a C-terminal succinimide. A second problematic residue was Asp, for which we observed some premature cleavage during initial binding to the Int^C resin. Moreover, RP-HPLC analysis of the eluted fractions upon thiolysis revealed two species with the molecular weight of the desired α-thioester. These results were not wholly unexpected, as Asp is known to cleave

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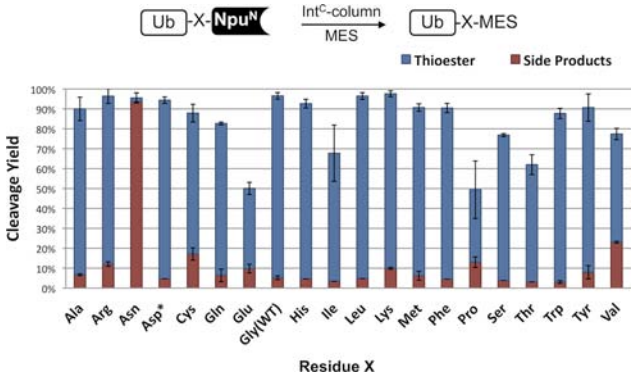

Figure 4. Effect of C-terminal amino acid identity on α-thioester formation. The 20 mutants of the protein Ub-X-Npu^N were expressed in *E. coli* varying the identity of the C-terminal amino acid of Ub (X) from the WT Gly to all other proteinogenic amino acids. Thiol-induced cleavage yields from the Int^C column were calculated from the SDS-PAGE analysis of the eluted fractions and left over resin beads. Ratios of α-thioester vs side products were determined from RP-HPLC and ESI-TOF MS analysis of the eluted fractions. The major competing reaction for all amino acids was hydrolysis with the exception of Asn for which its succinimide form was isolated instead. * See main text for a discussion on the problems associated with Asp. Error bars ± SD (n = 3).

prematurely from contiguous inteins through side-chain cyclization,[33,34] and its α-thioesters have been reported to migrate to the side-chain carboxylate yielding mixtures of α- and β-isomers.[35] These minor constraints aside, it is clear from these studies that our streamlined EPL system is compatible when the majority of amino acids are present as the last residue in the protein of interest.

Thioester Formation under Denaturing Conditions. Next we investigated if our system was compatible with denaturing conditions. Protein semisynthesis frequently requires the preparation of protein fragments, which are often poorly behaved and need to be purified in the presence of strong chaotropic agents. We first confirmed that the model protein Ub-Npu^N could bind the Int^C column in the presence of 2 and 4 M urea and that the corresponding Ub α-thioester could be generated with similar yields as obtained under native conditions (Figure S14). This is consistent with a previous study, which demonstrated Npu DnaE could splice in the presence of high concentration of denaturants.[27] We then turned to a more challenging target, namely a fragment of histone H2B (residues 1−116), a polypeptide that is prone to aggregation and difficult to generate as an α-thioester derivative using standard EPL procedures.[36] We expressed human histone H2B(1−116) fused to Npu^N in *E. coli*, extracted it from inclusion bodies in 6 M urea, and diluted it to 2 M urea prior to loading on the Int^C-intein column. The incubation was performed for 3 h at pH 6.0 to maximize binding while avoiding premature cleavage through hydrolysis. The pH was then raised to 7.2, and thiolysis was carried out for 36 h at rt (note that the presence of the denaturant slows down the thiolysis rate). Using these conditions, hH2B(1−116)-MES was obtained in excellent purity (>90% by RP-HPLC) and isolated yield (~20 mg per L of culture). This represents a significant improvement over previous protocols which afford less protein (4 mg per L of culture) and require the use of multiple chromatographic purification steps including RP-HPLC.[36] Importantly, the hH2B(1−116)-MES thioester obtained from the Int^C column could be directly used in EPL reactions without further purification. Accordingly, we successfully ligated the protein to a hH2B(117−125) peptide containing an acetylated Lys at position 120[37] to yield semisynthetic hH2B-K120Ac (Figure 5).

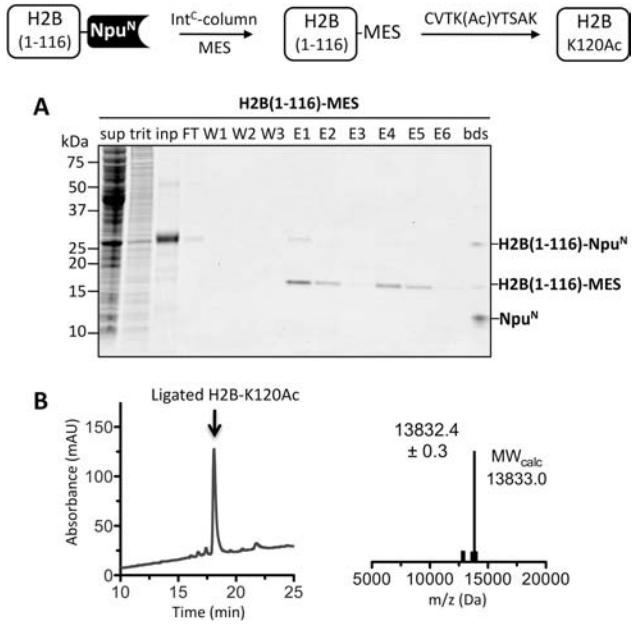

Figure 5. Semisynthesis of hH2B-K120Ac under denaturing conditions. (A) Coomassie stained SDS-PAGE analysis of hH2B(1−116) α-thioester generation in the presence of 2 M urea (sup: cell lysate supernatant, trit: 1% triton wash of the inclusion bodies, inp: solubilized inclusion bodies used as input for the Int^C column). E1−E3 were collected after 18 h of incubation with MES and E4−E6 after an additional 18 h. E1−E6 were pooled, concentrated to 150 μM, and ligated to the peptide H-CVTK(Ac)YTSAK-OH at 1 mM for 3 h at rt. (B) RP-HPLC (left) of the ligation reaction mixture and MS (right) of the ligated hH2B-K120Ac product.

Site-Specific Modification of a Monoclonal Antibody. Finally, we tested our streamlined EPL methodology for the modification of a monoclonal antibody. The site-specific modification of antibodies has become highly desirable in the area of biopharmaceuticals and diagnostics.[38,39] Currently, most commercially utilized methods to conjugate cargo to antibodies are relatively nonspecific and result in polydisperse mixtures that may vary from batch-to-batch. Since this heterogeneity can adversely affect both efficacy and safety of the conjugate, attention has turned toward technologies that afford site-specifically modified antibodies.[14,40−44] Indeed, protein semisynthesis via standard EPL and PTS has recently been used to generate monoclonal antibody conjugates with full activity.[14] Given this, we were eager to see whether our streamlined EPL process could be used in the facile generation of antibody conjugates. As a model immunoglobulin (IgG) for our studies, we used an antibody against the DEC205 receptor, a C-type lectin found predominantly on dendritic cells.[45] Accordingly, we designed a construct in which Npu^N was fused to the C-terminus of the heavy chain of the antibody (αDEC205-Npu^N). Initial expression tests of αDEC205-Npu^N in 293T cells resulted in very low levels of the antibody being secreted (Figure 6A). We have observed previously that the identity of the Int^N can have an effect on expression levels of its fusions.[28] Consequently, we asked whether we could obtain higher levels

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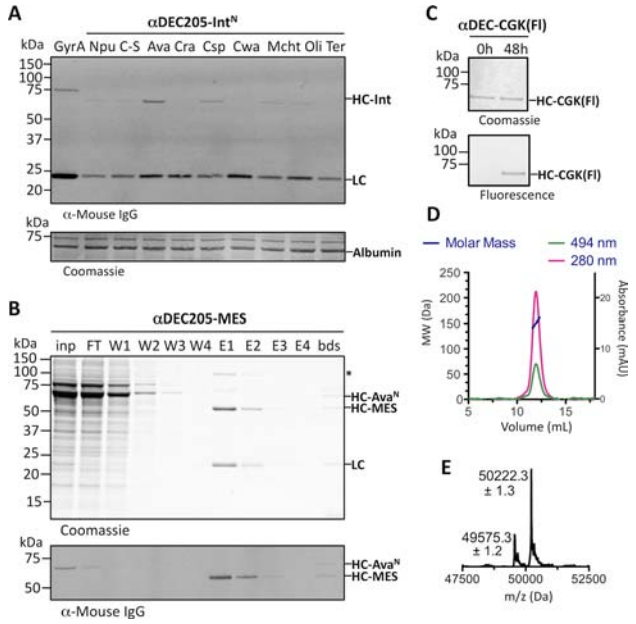

Figure 6. Purification of a monoclonal antibody α-thioester using a split intein and its site-specific modification. (A) Test expression of αDEC205 genetically fused to the contiguous Mxe GyrA intein and different split DnaE inteins through the C-terminus of its heavy chain (HC). Western blot of 293T cell supernatants of several αDEC205-Int fusions using an antibody against mouse IgG. A loading control is shown below. (B) Purification of αDEC205-MES thioester using the split intein column. (C) Elution fractions containing αDEC205-MES were concentrated to 20 μM and ligated to the CGK(Fl) fluorescent peptide at 1 mM for 48 h at rt. (D) SEC-MALS analysis of the ligated antibody showing that it retains its tetrameric structure after thiolysis and ligation (M_w = 151 kDa, M_w calcd = 148 kDa). (E) ESI-TOF MS analysis of degycosylated and fully reduced HC after ligation, showing 75% of the HC is labeled. Expected mass for ligation product = 50221.2 Da. Free HC = 49575.0 Da.

of secreted αDEC205-Int^N by varying the identity of the intein N-fragment. Several new αDEC205-Int^N constructs were generated in which Int^N corresponded to the N-fragment of a series of ultrafast split DnaE inteins, namely, Ava, Csp, Cra, Cwa, Mcht, Oli, and Ter. We also tested an Npu^N mutant (C-S) where the noncatalytic cysteines, Cys28 and Cys59, were mutated to Ser, to determine whether these residues influence secretion and maturation of the IgG tetramer (Figure 6A). Importantly, each of the Int^N fragments in this set can cross-react with the C-fragment of Npu without significant loss of splicing efficiency.[28] Thus, the Int^C column already in hand is compatible with all of these αDEC205-Int^N fusions. The contiguous Mxe GyrA intein was also fused to αDEC205 to test whether the use of N-intein fragments negatively affected expression levels compared to a full-length intein. An expression screen of this αDEC205-Int^N library was performed in 293T cells, revealing that the Ava^N and Csp^N fusions reproducibly exhibited higher expression levels than the other N-inteins, with the former being the best. Indeed, the expression levels of the αDEC-Ava^N construct were at least as good as the αDEC-GyrA construct. Based on this, the αDEC205-Ava^N fusion was chosen and purified over the Int^C column in an analogous manner to that of the soluble proteins described above (Figure 6B). Elutions from the column contained the thiolyzed αDEC205, which was subsequently ligated to the CGK(Fl) peptide (Figure 6C). Size exclusion chromatography (SEC) coupled to multiple angle light scattering (MALS) analysis confirmed that the antibody retained its tetrameric folded state after thiolysis and ligation (Figure 6D). We also performed MS analysis of the deglycosylated and fully reduced antibody, which confirmed the formation of a stable, nonreducible amide bond between the αDEC205 heavy chain and the fluorescent peptide with a 75% yield (Figure 6E). Importantly, we demonstrated that the semisynthetic αDEC-CGK(Fl) retains its ability to bind the DEC205 receptor to the same extent as a control αDEC205, previously shown to be fully functional *in vivo*[46] (Figure 7).

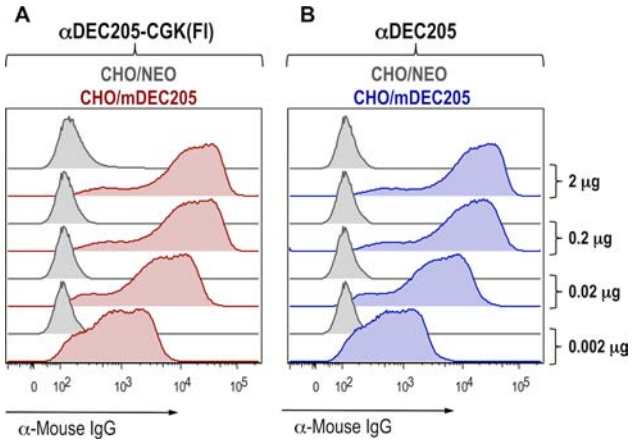

Figure 7. Binding of αDEC205-CGK(Fl) to the DEC205 receptor. (A) Dose dependent binding of αDEC205-CGK(Fl) to CHO cells expressing the mouse DEC205 receptor monitored by flow cytometry using a PE-labeled α-mouse IgG. Binding to control CHO/NEO cells, which do not express the receptor is shown in gray. (B) As in (A) but using a control α-DEC205 antibody.

Binding of αDEC-CGK(Fl) to the DEC205 receptor could be monitored not only by flow cytometry using an antimouse IgG secondary antibody (Figure 7) but also through the site-specifically incorporated fluorescein (Figure S20).

CONCLUSION

We have shown that split DnaE inteins can be engineered for the efficient generation and isolation of protein α-thioesters. Furthermore, the strategy can be seamlessly integrated with EPL, and one pot purification/ligations can be performed without isolation of the α-thioester intermediates. We note, however, that the ligation step (i.e., NCL) still requires the use of high concentrations (high μM or above) of N-terminal cysteine peptide for efficient reactions. The strong and specific interaction between the two intein fragments facilitates the purification of protein α-thioesters under a variety of conditions, including strong denaturants, and the isolation of these products directly from cell lysates proceeds significantly faster than via many mainstream EPL strategies, which often take multiple days and several intermediate enrichment/purification steps. Additionally, while split intein fusions are usually associated with low levels of expression, we show here that certain DnaE Int^N fragments express to the same levels as commonly used contiguous inteins when fused to a monoclonal Ab. Moreover, the absence of premature cleavage events allowed us to generate semisynthetic proteins, such as H2B-K120Ac in far superior yields than those of previously established protocols. Importantly, we have shown the utility

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of this methodology for the modification of complex biomolecules such as an IgG. Thus, streamlined EPL via our split DnaE intein column should provide an efficient route to site-specifically modified proteins for basic biochemical research as well as translational applications.

EXPERIMENTAL SECTION

Preparation of Int^C Column. Npu^C-AA-Cys-OMe peptide (0.5 μmols per mL of resin) was dissolved in 2 column volumes (CV) of coupling buffer (50 mM Tris·HCl at pH 8.5, 250 μL for 125 μL resin) and treated with 25 mM TCEP from a 1 M stock for 15 min. The peptide solution was then added to 1 CV of agarose SulfoLink resin (from Pierce, loading: 18.4 μmol iodoacetyl groups/mL of resin) in a small fritted column and incubated for 15 min on a nutator, followed by 30 min standing at rt. The column flow-through was collected, and the column was washed twice with 2 CV of coupling buffer. Unreacted iodoacetyl groups on the resin were blocked by a treatment with 50 mM H-Cys-OMe in coupling buffer for 15 min on a nutator, followed by 30 min standing at rt. Column was washed twice with 1 CV of coupling buffer, 2 CV of 1 M NaCl, and finally 2 CV of water. Int^C columns were stored in storage buffer (100 mM phosphate, 150 mM NaCl, 1 mM EDTA, 0.05% NaN_3, pH 7.2), at 4 °C for up to 2 weeks.

Purification of POI-MES α-Thioesters from Cell Lysates Soluble Fraction. *E. coli* BL21(DE3) cells transformed with the desired POI-Npu^N plasmid were grown in 1 L of LB containing 100 μg/mL of ampicillin at 37 °C until OD_{600} = 0.6. Protein expression was induced by addition of 0.5 mM IPTG. After harvesting the cells by centrifugation (10 500 rcf, 30 min), the cell pellets were transferred to 50 mL conical tubes with 20 mL of high-salt binding buffer (100 mM phosphate, 500 mM NaCl, 1 mM EDTA, 1 mM TCEP, pH 7.2) with complete protease inhibitor cocktail (Roche) and stored at −80 °C. Resuspended cells were lysed by sonication, and the soluble fraction recovered by centrifugation (17 000 rcf, 10 min). Onto 62.5 μL of Int^C column, 300 μL of the soluble fraction were loaded and incubated at rt for 5 min. After incubation, the flow-through was collected, and the column was washed with 300 μL of high-salt binding buffer, 300 μL of wash buffer (100 mM phosphate, 300 mM NaCl, 1 mM EDTA, 1 mM TCEP, pH 7.2), and 300 μL of binding buffer (100 mM phosphate, 150 mM NaCl, 1 mM EDTA, 1 mM TCEP, pH 7.2). The column was capped and incubated with 150 μL of elution buffer (100 mM phosphate, 150 mM NaCl, 200 mM MES, 10 mM TCEP, 1 mM EDTA, pH 7.2) for 18 h. Flow-through was collected, and the column was washed three times with 150 μL elution buffer.

One-Pot EPL using Int^C Column. Onto 62.5 μL of Int^C column, 300 μL cell lysate containing Ub-Npu^N was loaded and incubated as described before. After incubation, the column flow-through was collected. The column was washed with 300 μL high-salt binding buffer, 300 μL wash buffer, and 300 μL binding buffer. The column was capped and incubated with 75 μL EPL buffer (100 mM phosphate, 150 mM NaCl, 200 mM MES, 50 mM MPAA, 1 mM EDTA, 10 mM TCEP, pH 7.9) containing 1 mM CGK(Fl) peptide for 18 h. The flow-through was collected, and the column was washed three times with 75 μL elution buffer. All fractions were analyzed by SDS-PAGE. Gels were first imaged using the green fluorescence channel on a GE ImageQuant LAS 4010 imager and then coomassie stained.

Preparation of αDEC205-CGK(Fl) via Streamlined EPL Using the Int^C Column. HEK293T cells were transiently cotransfected with antimouse-DEC205-LC and antimouse-DEC205-HC-Ava^N using lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Typical cotransfections were performed in 10 cm plates. After 4 days incubation at 37 °C with 5% CO_2, cell supernatants were harvested and spun down at 2000 rcf for 20 min at 4 °C, filtered through a 0.22 μm filter, and supplemented with complete protease inhbitors. For a typical purification, 50 mL of αDEC205-Ava^N transfected cell supernatants were concentrated to a final volume of 5 mL and exchanged into binding buffer. The resulting Ab solution (input) was applied to an Int^C column of 300 μL beads (loading: 1.8 μmol NpuC peptide/mL) and incubated at rt for 30 min. Column flow-through was collected, and column washed three times with 3 CV of wash buffer and once with 3 CV of binding buffer. The column was capped and incubated with 3 CV of Ab elution buffer (100 mM phosphate, 150 mM NaCl, 200 mM MES, 1 mM TCEP, 1 mM EDTA, pH 7.2) for 20 h. The column flow-through was collected, and the column washed three times with 3 CV of Ab elution buffer. Elutions containing αDEC205-MES were combined and concentrated down to 20 μM. Ligation was initiated by addition of 1 mM CGK(Fl) peptide and 1 mM TCEP and adjusting pH to 7.5−8.0. The reaction was incubated in the dark at rt for 48 h and monitored by SDS-PAGE imaged using a fluorescence scanner and coomassie staining. Once the reaction was completed, the ligated Ab was diluted to 200−500 μL and dialyzed into 100 mM phosphate, 150 mM NaCl, 1 mM EDTA, 1 mM TCEP, pH 7.2.

ASSOCIATED CONTENT

Supporting Information

Full experimental details including materials and methods, cloning, protein purification, and characterization, calculation of thiolysis and recovery yields, Int^C column regeneration protocols, and antibody characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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